MYCORRHIZA DEVELOPMENT IN TREES FOR REVEGETATION OF ERODED MOUNTAIN SLOPES

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FRONTISPIECE Lyndon Hill; Craigieburn Range (1,260 metres).

South face showing extent of erosion. Gullying and broad shingle screes are characteristic of the high country slopes in this locality. Trial plots of exotic species can be seen above the native beech tree line.

LYNDON HILL; Craigieburn Range.

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ABSTRACT

The ectomycorrhizal association between Mountain Pine (*Pinus mugo*) and the fungus *Suillus luteus* has been studied in an attempt to promote establishment and growth of trees on high altitude revegetation sites.

In vitro growth studies of *S. luteus* showed it was capable of growing at low phosphorus levels and utilising a variety of carbohydrate substrates. A number of organic acids, components of root exudates, stimulated fungal growth.

Basidiospores have the greatest potential for pine seed inoculation. Investigation of the viability, storage and germination of these spores revealed that frozen or cool stored spores and freeze dried hymenial tissue were the best forms of inocula. Basidiospore viability was best determined by acridine orange staining with fluorescence microscopy. Nicotinic acid and inositol stimulated spore germination although germination levels were always low. Large numbers of basidiospores were necessary to obtain good mycorrhiza formation on seedlings and the inocula lost effectiveness with long storage periods.

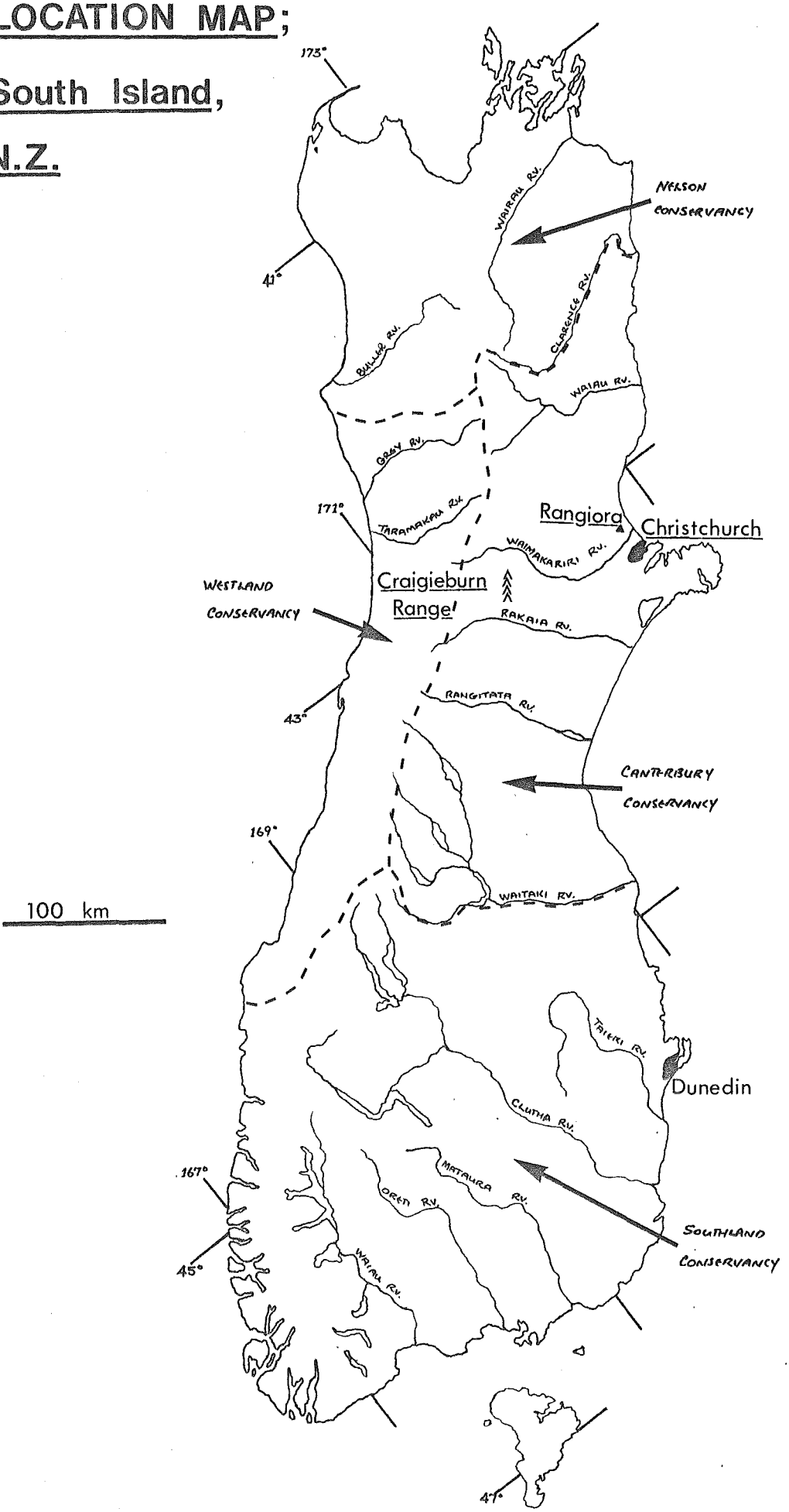
A scanning electron microscope study of the mycorrhizal infection process showed the presence of novel 'vesicular bodies', closely associated with the Hartig net.

Granulated seeds containing a basidiospore inoculum were prepared and seedlings developing from these possessed low levels of mycorrhizas.

LOCATION MAP;

South Island,

N.Z.



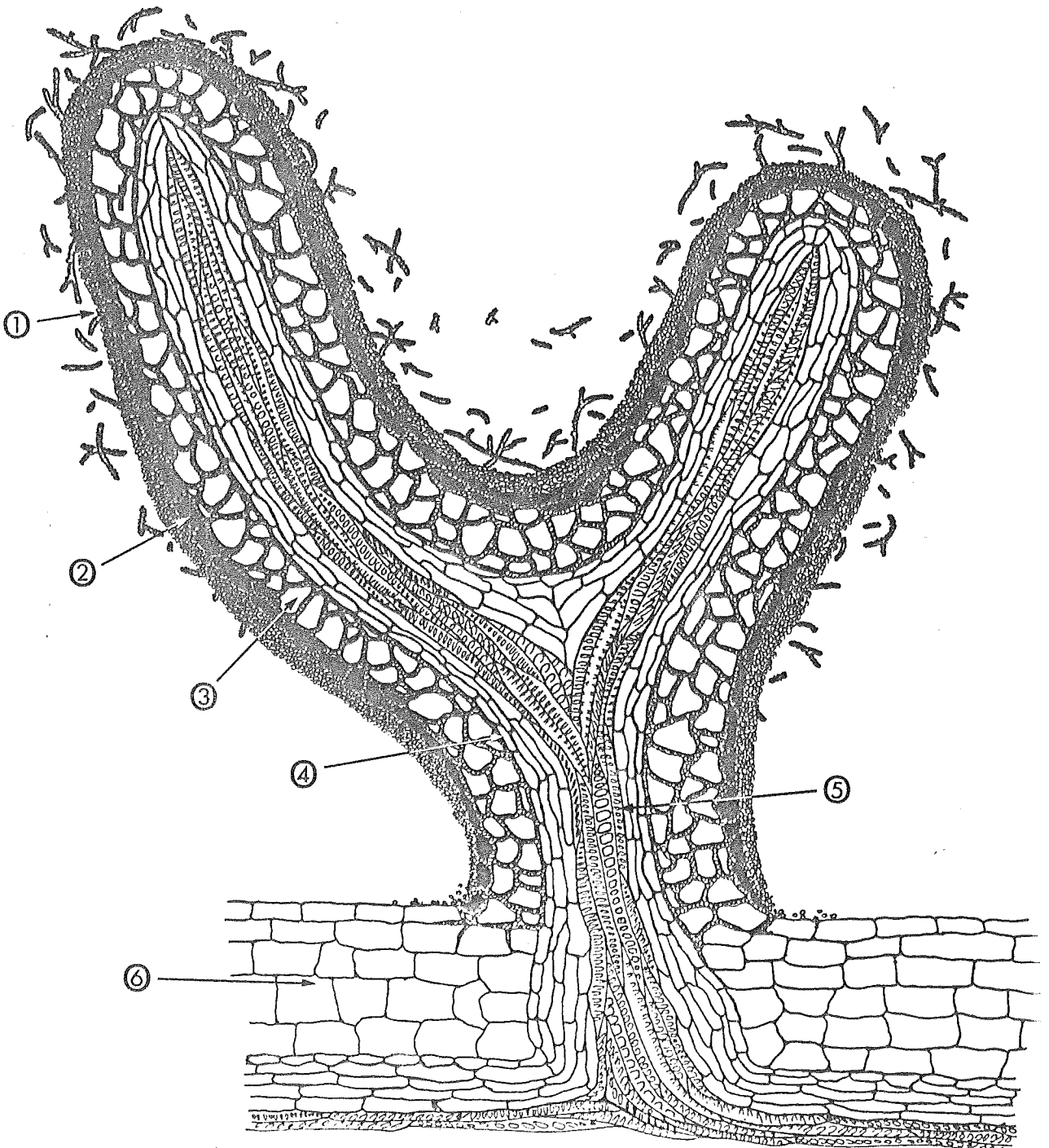
CHAPTER I

GENERAL INTRODUCTION

Under natural conditions, many plant species form symbiotic relationships with fungi known as mycorrhizas. This study is primarily concerned with the development of techniques for inoculation of pine seeds with mycorrhizal symbionts. These are to be used for the direct seeding of trees in New Zealand's high country areas as part of an erosion control project initiated by the New Zealand Forest Research Institute (Protection Forestry Division). The approach adopted in this work was based on the consideration that "failure in inoculation programmes will arise from an inability of the fungus to colonize roots and it will be necessary to select fungi not only on their ability to stimulate growth but also to colonize the root and infect the plant rapidly." (Bowen *et al.* 1971)

The first recognition of the mycorrhizal habit was made in the late 19th Century by Frank (1885) and since then extensive studies have shown its importance particularly where growing conditions are marginal. With forest trees the ectomycorrhizal form of association is most common. Ectomycorrhizas form a distinct sheath of hyphae on the root surface (Fig. 1.1) and hyphae penetrate into the epidermis and cortex of the root to form an intercellular network known as the Hartig net. Mycelial strands and rhizomorphs may be

1-1
TYPICAL P. mugo ECTOMYCORRHIZA.



1. Fungal Sheath
2. Tannin Cells
3. Cortex - Hartig Net
4. Endodermis
5. Vascular Tissue
6. 'Mother' Root

0·5 mm

produced, substantially increasing the soil volume available to the host/mycorrhiza. Many other plants form endomycorrhizal associations where the sheath is usually lacking and intra-cellular hyphal projections, vesicles or arbuscles, are the predominant feature. Endomycorrhizas are uncommon in forest trees, although an intermediate form, the ectendomycorrhiza, may be found. The short roots of most coniferous species usually form an ectomycorrhizal association with members of the larger basidiomycetes (the Hymenomycetes and Gasteromycetes). Their fruiting bodies are evident in many forest systems and their role in litter degradation and nutrient mobilization is well documented (Bowen, 1969; Harley, 1969).

Afforestation of eroded mountain slopes in high country areas of New Zealand's South Island presents many problems of adaptation to extreme climatic and edaphic factors. Much of the work to date has been carried out by the New Zealand Forest Service's Forest and Range Experimental Station (Rangiora) at experimental plots in the Craigieburn Range/Broken River area of Canterbury. (Fig. 1.2). Many seedlings do not develop mycorrhizas even after the first season's growth which severely retards their progress and seedling establishment is also very difficult (Nordmeyer, 1965). This is due to the instability and low nutrient status of the soil, combined with large diurnal and seasonal temperature fluctuations. To improve establishment and growth of young trees, an early symbiotic association must be attained in the exotic pine species used for afforestation. Pine seedlings can be inoculated in nursery plots but the

FIGURE 1.2 A Lyndon Hill, East face.

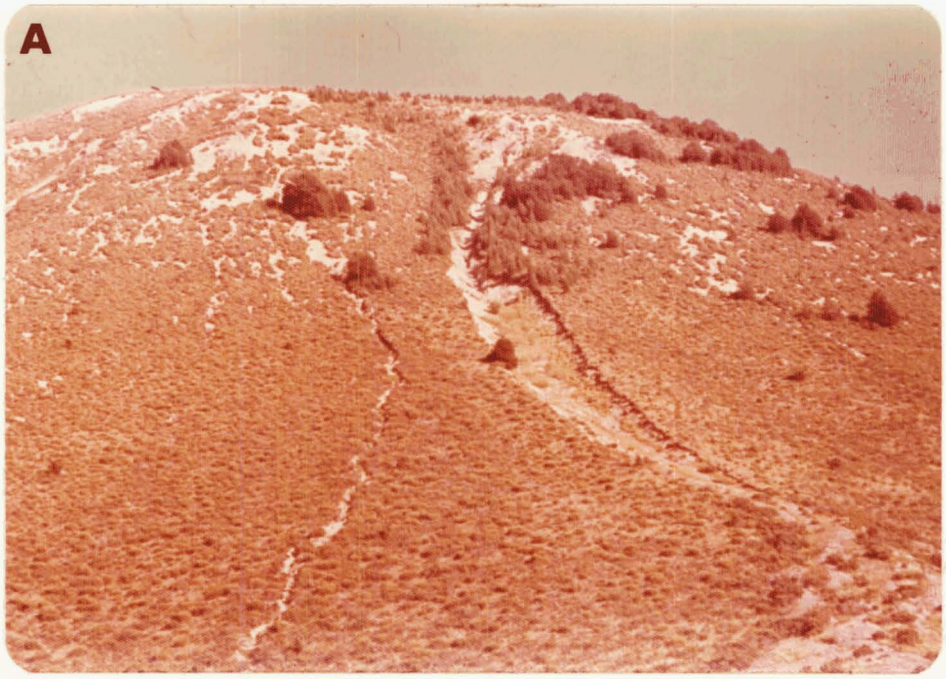
The large gully is being stabilised with pines near the hill top and willow (Salix) species lower down. All of the areas of broken ground visible are now planted with young pines.

B Lyndon Hill.

Experimental plots of pine, larch (Larix) and spruce (Picea).

FIGURE 1.3 Sporophores of Suillus (Boletus) luteus growing amongst pines shown in Fig. 1.2 A and B.

1-2 A



B

1-3

procedure is labour intensive and often results in inefficient mycorrhizal associations being established. The soils of these high country areas are yellow brown earths and have only thin organic horizons if stable. In many instances the subsoil is exposed and the natural mycorrhizal symbionts are absent. The most promising form of inoculum for direct sowing would appear to be basidiospores (Bowen, 1969; Marx, 1976; Theodorou and Bowen, 1973). Little, however, is known about factors affecting their preservation, germination and consequent invasion of the host root.

The association between Mountain Pine (Pinus mugo, Turra) and Suillus (Boletus) luteus (L. ex Fries) was investigated. Mountain Pine is found to grow successfully under New Zealand conditions up to 1600 metres above sea level. It is resistant to wind and snow pack damage, growing well on dry slopes and having a low spreading canopy making it suitable for erosion control. S. luteus is believed to be the main mycorrhizal fungus associated with Pinus species at higher altitudes in New Zealand. Some sporophores of S. elegans are also found, but are closely associated with Larix species. S. luteus produces an abundance of sporophores enabling ready basidiospore collection (Fig. 1.3) and was used in this study because of its predominance in these areas and its known mycorrhizal habit with P. mugo (Trappe, 1962).

Techniques for preservation of spores and for determining their viability were important elements of this

study because practical methods of seed inoculation tested by the Forest and Range Experimental Station had failed to produce mycorrhizal seedlings, thus bringing basidiospore viability into question. A further consideration was to provide both seed and spores with a suitable environment, such as a coating, to ensure viability in the harsh conditions.

Many factors are known to influence germination of spores and mycelial growth of basidiomycetes (Fries, 1966; Watling, 1971; Losel, 1964, 1967). An essential component of this study was to determine optimal growth conditions of S. luteus and investigate the possibility of chemical stimulation of basidiospore germination. Host root exudates are known to stimulate spore germination and some components of these were studied for their effects.

The morphological interactions of host and symbiont were observed at different stages of development, using naturally infected seedlings and those raised in aseptic or "semi-aseptic" culture, with both the light and scanning electron microscopes.

CHAPTER II

LITERATURE REVIEW

2.1 INTRODUCTION

The mycorrhizal habit, in its diverse forms, has been studied intensively since the concept was first accepted in the late 19th Century. At first the idea of a 'fungus-root' was the subject of much conjecture as many workers considered the association to be a parasitic one. Later observation and study by Hatch (1937) showed that this view was erroneous. The morphological and physiological interactions between host and fungus have been closely studied as reviewed by Harley, 1969; Hacskaylo, 1969; Marks and Kozlowski, 1973. These studies have shown the importance of mineral mobilisation and nutrient exchange, hormonal balance, and light to the mycorrhizal associates.

The processes of exudation by host roots and fungus and the consequent interactions between the mycorrhizal fungi and other elements of the rhizosphere are very important to the understanding of the ectomycorrhizal association. They determine the type of mycorrhiza that is established and benefits gained by both partners. In a recent review, Trappe (1977), outlined the factors to be considered when selecting ectomycorrhizal fungi for nursery inoculation.

Many of these also apply to the direct seeding situation and their significance to afforestation has been reviewed by Bowen (1969), Bjorkman (1970), Bowen *et al.* (1971) and Slankis (1974).

The literature covered in this chapter indicates the wide interest in mycorrhizal inoculation research internationally and the advances currently being made in mycorrhizal studies.

2.2 HISTORY OF MYCORRHIZAL STUDY

The origins of mycorrhizal study date back to the mid 1800's. Observations on the achlorophyllous Pine-sap (*Monotropa hypopitys*) led to the conclusion by Rylands (1842) that the roots were associated with fungal tissue (it has been shown that this member of the wintergreen family is saprophytic, obtaining additional nutrients from pine trees by way of their mycorrhizas. The mycorrhiza thus has a host and a parasite to maintain, Bjorkman, 1960). Boudier (1876) noted that only peripheral cortical cells of *Elaphomyces* were penetrated by the mycorrhizal mycelium and that the growth of infected roots was stimulated. The intercellular fungal network was recognised by Hartig (Th.) and illustrated in his "Anatomie and Physiologie der Holzplanzen" (1878) but the function of this "Anastomosirenden Geflicht" was never fully comprehended. Many workers had observed that root hairs were only prevalent on young tree seedlings, never on older trees and dichotomy in pine roots was known to be associated with the infected state. Thus the association between fungus and root had been widely

reported, but it was not until publication of A.B. Frank's paper (1885) that this was given the specific terminology "Mycorhiza"; "Der ganze Körper ist also weder Baumwurzel noch Pilz allein, sondern ähnlich wie der Thallus der Flechten, eine Vereinigung zweier verschiedener wesen zu einem einheitlichen morphologischen organ, welches viellecht passend als Pilzwurzel, Mycorrhiza, bezeichnet werden Kann" (l.c. pp 129, see also Kelly, 1931).

This paper aroused considerable opposition. Hartig (Rbt.) and others could not accept that mycorrhizas were beneficial to the host or were of common occurrence. Frank, meanwhile, published another paper in 1887 describing endotrophic mycorrhizal roots. By the turn of the century, however, the mycorrhizal habit was largely accepted (Gregory, 1889) and emphasis was shifted to the causes and effects of the association. Stahl (1900) proposed his Mineral Salt Theory which stated that in situations where plant transpiration was restricted and soils were infertile, mycotrophy was induced. The mycorrhizas increased the transpiration rate and thus aided the release of salts from humic soils.

Frank (1894) realised that forest trees and soils were deficient in nitrates and believed that nitrogen was assimilated as ammonium salts or in organic form. Only when nitrates were not available, according to Frank, did mycorrhizas benefit trees by utilizing humic nitrogen. Because of the increased interest in mycorrhizal habits, particularly of conifers, much work was published describing these associations

and their effects on the symbionts (Melin, 1925; Hatch, 1937; Mitchell et al, 1937; Rayner, 1934, 1936, 1939; Bjorkman, 1942).

Melin (1917) at first favoured Frank's theory. He had observed that in soils where nitrogen mobilisation was high, few mycorrhizas occurred and conversely that only mycorrhizal trees grew well in low nitrogen bog soils. In 1925, Melin considered that pH, acidic in humic soils, allowed optimum fungal development. He demonstrated that under aseptic conditions known mycorrhizal associations could be produced and that fungal stimulating substances were produced by germinating pine seeds. These are now known to be growth substances such as thiamine (Melin and Lindeberg, 1939; Melin and Nyman, 1940, 1941; Melin and Norkrans, 1942, 1948). In 1927, however, Melin conducted experiments showing an increase in fungal and tree growth related to increased nitrogen mobilisation in the soil, thus contradicting Frank's theory.

Some still believed that the mycorrhizas were of no benefit to the trees (McDougal, 1914, 1922; Moller, 1947) but Bjorkman (1937, 1940, 1942) disproved this, showing their importance in forest systems. Inoculation experiments were carried out both on soils thought to be devoid of suitable mycorrhizal fungi (Hatch, 1936, 1937; Young, 1936; Rayner, 1938) and on soils deficient in some essential nutrient (Mitchell et al, 1937; Miller, 1938; McComb, 1938). It was shown that mycorrhizas increase nutrient uptake and are not primarily concerned with the utilization of organic nitrogen. Evidence that other factors also affected the symbiotic

association was given by Gast (1937), who found that lowered light intensities inhibited mycorrhizal formation.

Kessel (1927) described the dependence of some pine species on a "biological soil factor". The association between pines and mycorrhizal fungi, mainly Boletus species, was recorded by many workers (Melin, 1923; Young, 1936; Hatch and Doak, 1933; Hatch and Hatch, 1933; Doak, 1934; Addoms, 1937). Various studies of conifer mycorrhizas were reported by McDougall (1928), McArdle (1932) and How (1940). Romell (1938) observed that mycorrhizal fungal sporophores do not develop unless there is a direct connection to the host tree (with the exception of B. subtomentosus, known to have saprophytic habits - Romell, 1939; Modess, 1941). It was known that mycorrhizal fungi depend on soluble carbohydrates (Melin, 1925; Romell, 1939) and this ultimately led Bjorkman (1942) to propose his carbohydrate theory of mycorrhizal development. This related mycorrhizal infection to the photosynthesis status of the plant; high light levels increase carbohydrate release and thus promote infection. Lundegardh and Stenlid (1944) observed that fungal growth towards tree roots was stimulated by excreted substances, including carbohydrates (Norkrans, 1944). MacDougal and Dufrenoy's (1944) findings that excised pine mycorrhizas can survive in forest soil for considerable periods indicate that the fungus must manufacture some carbohydrates and thus does not support Bjorkman's theory. Rayner and Neilson-Jones (1944) demonstrated that toxic substances in a moorland soil can adversely effect mycorrhizal initiation, thus there are many factors affecting mycorrhizal formation.

The concept of mycorrhizal formation is therefore varied. The ideas of Hatch, that deficiency of one or more essential major nutrients is an important factor in promoting infection and Bjorkman, that adequate photosynthetic levels are necessary, must both be considered to influence formation although their degree of involvement is difficult to determine. Hormones and growth factors are now also known to have important roles in mycorrhizal formation. The literature from this period on has been widely reviewed, including Harley, 1969 and Marks and Kozlowski, 1973, covering many fields of mycorrhizal study and the pertinent literature is considered in following sections of this chapter.

2.3 STRUCTURE AND FUNCTION OF MYCORRHIZAS

2.3.1 Mycorrhizal Morphology

Mycorrhizas are a common feature of many plant root systems. Two basic types are found - the ectotrophic and endotrophic mycorrhizas - but because of the multiplicity of associations, various modifications of these are seen. Ecto- and endo-mycorrhizas (Peyronel *et al.* 1969) were first described by Frank (1885, 1887) and comprise the majority of symbiotic associations found, but the pseudomycorrhizas and ectendotrophic mycorrhizas described by Melin (1917) also occur. The system of biotrophic classification proposed by Lewis (1973) should be considered here as well, in which case ectomycorrhizas are more correctly termed sheathing mycorrhizas.

The ectendomycorrhizas exhibit a thin sheath (in some

cases completely lacking) with tannin filled host cells in the outer cortex (Mikola, 1965, Laiho, 1965). The Hartig net tends to be coarse with hypertrophied cells in the inner cortex where the hyphae may be intercellular and intracellular. These hyphae are often bulbous (Mikola, 1965; Hofsten, 1969) and bear a resemblance to vesicular structures discussed in Chapter VIII of this thesis. Laiho (1967) and Wilcox and Ganmore-Neumann (1974) showed that seedlings with ectendomycorrhizas grow better than uninfected ones. The ectendomycorrhizal associations of Pinus resinosa have been studied in detail by Wilcox (1968, 1971) and Wilcox et al. (1974). It has been suggested (Marks and Foster, 1967) that the intracellular penetration may occur after old ecto-mycorrhizal cortical cells degenerate and it is also known that a tree species' roots may associate with several symbiotic fungi (Zak and Marx, 1964; Lamb and Richards, 1970). Thus, when considering inoculation of trees with mycorrhizal fungi, it is possible (even likely) that a host may change its symbiont several times during maturation or alter the structure of its existing symbiont. Mycorrhizal symbionts must therefore be carefully selected.

The structure and associations of endotrophic mycorrhizas have been widely reviewed by Harley (1969), Mosse (1973) and Gerdemann (1975). As these are of minor importance in forest trees, they are not covered in detail here (see Sanders et al. 1975).

Various studies have been made of the structure of ectomycorrhizal roots. Robertson (1954), Foster and Marks

(1966), Wilcox (1968) and more recently Strullu and Gourret (1973) studied the morphology of Pinus mycorrhizas. Eucalypt mycorrhizas have also been studied by Chilvers and Prior (1965), Chilvers (1968), Tippet and O'Brien (1976) and Ashton (1976). The ultrastructure of ectomycorrhizal roots as revealed by the transmission electron microscope is discussed by Marks and Foster (1973). Little use has been made by these or other workers of the scanning electron microscope (S.E.M.) to elucidate the morphological characteristics of the symbionts although Kinden and Brown (1975, 1975a, 1976) have used the S.E.M. to advantage in a study of vesicular arbuscular mycorrhizas of yellow poplar. Their techniques, with some modifications, were used in this study (Chapter VIII). Strullu and Gourret (1973) presented micrographs of P. brutia mycorrhizal dichotomies which show the extensive hyphal weft over the root surface, although the hyphae appear distorted due to dehydration that has occurred during their preparation for microscopy. Strullu (1973, 1974, 1976) has also used the S.E.M. to study ectomycorrhizas of Pseudotsuga menziesii, but no details of the intracellular structure are shown.

The S.E.M. could be used to study many mycorrhizal systems and give more information about the spatial relationships between host and fungus. If combined with immunofluorescent studies described by Unger and Wagner (1963), Preece and Cooper (1969), Warnock (1973) and Schmidt et al. (1973), it may be a useful tool in the study of mixed mycorrhizal associations.

2.3.2 Mycorrhizas and the Rhizophere

The S.E.M. is now becoming widely accepted in the study of rhizophere systems. Harley (1948) discussed the soil ecology of mycorrhizas, stressing the variability of the rhizophere as affected by the particular soil/root system. Studies in tree systems using conventional agar plate isolations were done by Katznelson *et al.* (1962) and Parkinson *et al.* (1967, 1968, 1969, 1919a). Rovira *et al.* (1974) found light microscopic estimates of rhizoplane microflora to be more precise than plating techniques and bacterial distributions could also be plotted (Newman and Bowen, 1974). Foster and Marks (1967) used the T.E.M. in a study of the *Pinus radiata* rhizosphere, finding specific types and distributions of micro-organisms. The T.E.M. has also been used by Greaves and Darbyshire (1972) and Guckert *et al.* (1975) to study the mucigel layer of plant roots and can give information on the *in situ* relationship of micro-organisms to the soil and root surfaces (Foster and Rovira, 1972; Waid, 1973).

The S.E.M. has advantages over the T.E.M. because of simple specimen preparation and good depth of focus over its magnification range. By critical point drying and low-temperature freeze drying, Campbell and Rovira (1973) obtained micrographs showing little distortion and disturbance of the rhizosphere. Bacteria and fungal spores usually survive the preparation and electron beam well, but hyphae are often distorted. Todd *et al.* (1973) retained the hyphal structures successfully, but gave no indication of

the methods used to prepare their specimens. Rovira and Campbell (1974, 1975) extended their studies of the wheat root rhizosphere and showed that bacterial numbers increased in roots colonised by the 'take-all' pathogen, Gaeumannomyces graminus, eventually causing lysis of the fungal hyphae. Old and Nicholson (1975) used the S.E.M. to locate micro-organisms on dune grass roots and gave evidence for bacterial penetration and colonisation of cortical cells. They also detected several phases of V.A. mycorrhizal development on these roots.

Thus the S.E.M. is an important tool in the study of the rhizosphere and of the development of mycorrhizal associations. Careful preparation methods will allow detailed in situ studies of micro-organisms at the plant root soil interface.

2.3.3 Host-Fungus Interactions

There are many interesting interactions between the mycorrhizal fungus and the higher plant. Perhaps the most intriguing of these is the aspect of host invasion. What factors allow fungal hyphae to penetrate the host root tissues and confine it? It is now thought that the early stages of root invasion are of a parasitic nature, with the balanced symbiotic state being achieved by limited host production of specific phytoalexins (Hacskaylo, 1972; Marx, 1973; Ubrizy, 1976) and polyphenolic tannins (Foster and Marks, 1967; Hillis and Ishikura, 1969; Ling-Lee et al. 1977) thus preventing further fungal invasion (pathogenicity). This is, therefore

an example of the alleloparasitism of Meyer (1974) or the obligate biotrophy of Lewis (1973).

Interactions important in the establishment and functioning of mycorrhizas are briefly reviewed.

2.3.3.1 Mycorrhizal Effects on Host Root Pathogens

In the forest system, work by Marx (1969, 1970) and Marx and Davey (1969) has shown that three important mycorrhizal symbionts, Laccaria laccata, Pisolithus tintorius and S. luteus, inhibit growth of many pine root pathogens, as do some Lactarius species (Park, 1970). L. laccata is effective in protecting Douglas Fir against Fusarium root rot (Stack and Sinclair, 1974). Certain other Suillus species, S. variegatus (Krupa and Nylund, 1972), S. placidus (Froidevaux and Amiet, 1974) and S. granulatus (Richard and Fortin, 1975) are also known to antagonise conifer root pathogens. Thus, if specific mycorrhizal fungi are selected and used to inoculate trees, they may protect against root diseases as well as stimulating growth (Zak, 1964; De la Cruz and Hubbell, 1975).

2.3.3.2 Mycorrhizal Fungal Exudates

Many substances are produced by the fungal associate, some of which help mycorrhizal initiation. Lindeberg (1948) has shown the presence of polyphenol oxidase for enzymatic breakdown of lignins, not only in litter decomposing basidiomycetes, but also in some mycorrhiza formers such as B. subtomentosus and several Lactarius species. Cellulases are also implicated in the invasion of fungal elements

into the host roots (Clowes, 1954, Hacskaylo, 1973).

Santoro and Casida (1962) noted the production of antibiotics of the polyene type, affecting root pathogenic micro-organisms, by several mycorrhizal fungi including Amanita rubescens, A. caesaria, A. muscaria, B. bicolor and B. luteus. Those extracted from B. luteus were particularly active, confirming Marx's findings (Section 2.3.3.1). B. variegatus is known to produce volatile organic compounds (Krupa and Fries, 1971) and in association with Scots pine roots, the concentration of these terpenoid compounds was shown to increase several fold. Many of these terpenes have known fungistatic properties.

Auxins appear to be important in determining mycorrhizal host morphology, such as the formation of root dichotomies. They are produced by many mycorrhizal Boletus species (Ulrich, 1960, 1960a) and are capable of inducing morphological changes in pine roots in vitro (Slankis, 1950, 1958). Turner (1962) showed that mycorrhizal fungi differ in their ability to induce dichotomies in pine roots. They can therefore exert limited control over host growth processes, depending on how much auxin the particular mycorrhizal fungus produces. Ritter (1968) indicated that mycorrhizal fungi can produce inhibitors of indole acetic acid (IAA) oxidation, so inducing hyperauxiny of host roots. Conversely Fortin (1967) has shown that several mycorrhizal basidiomycetes are inhibited in growth by IAA but when considering their interaction with pine roots "la racine en accumulant l'AIA favorise la croissance du champignon en prevenant l'autoinhibition de son mycelium par l'AIA qu'il produit", (Fortin, 1970). Lateral development in P. radiata

has recently been studied in relation to the fungus-host hormonal interactions by Wilson (1975), showing that naphthalene acetic acid (NAA) induces dichotomy in small diameter, higher order roots. The influence of auxins is thus very diverse and hormonal effects are of undoubted importance to both host and symbiont but, as indicated by Slankis (1973) these need to be studied in more detail.

2.3.3.3 Host Root Exudates

The effect of root metabolites on mycorrhizal fungi is an interaction of importance to both the breaking of spore dormancy and the chemotaxic attraction of hyphae to host roots. Melin (1954, 1963) and Melin and Rama Das (1954) investigated the growth factor requirements of mycorrhizal fungi and the influence of root metabolites on them. Further investigation into plant root exudates was carried out by Rovira (1956, 1965, 1969) and Rovira and Harris (1961). This has since been extended to a study of exudation zones on plant roots and their association with rhizosphere micro-organisms (Rovira, 1973). Melin and Krupa (1971) and Krupa et al. (1973) have also studied the occurrence and effects of volatile organic root constituents on mycorrhizal fungi which in many cases inhibit their growth.

The kinds of root exudates from various trees and their importance to mycorrhizas are reviewed by Schroth and Hildebrand (1964) and Bowen and Theodorou (1973). Smith (1970) showed abundant carbohydrates to be present in young maple seedlings, a factor which may aid in initiation of mycorrhizal associations. Mature trees release more amino acids/amides and organic acids (Slankis et al. 1964). It appears that

inhibitory substances are produced in old, secondarily thickened axes and stimulatory ones are produced in the primary rootlets. Melin (1954) postulated the presence of a stimulatory "M-factor" produced by plant roots essential to the growth of mycorrhizal fungi. Although not chemically defined, the "M-factor" is produced by many plant species including those not forming ectotrophic mycorrhizas (Theodorou and Bowen, 1971).

In summary, "a combination of factors emanating from the host, such as simple carbohydrates, vitamins, amino acids and "M-factor" together with inhibitory substances tends to select the mycorrhizal fungi which themselves produce substances that change the metabolism and morphology of the host", (Harley and Lewis, 1969).

2.3.4 Mycorrhizal Physiology

The basis of the physiological interaction between the different organisms of the mycorrhiza have been studied by several workers and aspects of this are reviewed by Melin (1953), Harley and Lewis (1969) and Meyer (1974). Some of the work covered in this thesis is concerned with the effects of micro-requirements, carbohydrate sources and phosphorous/nitrogen nutrition on the growth of S. luteus and germination of its spores.

2.3.4.1 Micro-requirements

Mycorrhizal fungi have complex requirements and as a consequence are difficult to grow and maintain in pure culture. Stevens (1974) describes various media giving

adequate growth of these fungi *in vitro*.

Melin *et al*. (1939, 1942, 1940, 1941), Mikola (1948) and Norkrans (1950) have shown the vitamin requirements of several mycorrhizal fungi, particularly *Boletus*, *Lactarius* and *Tricholoma* species. In pure culture on synthetic media, all proved to be deficient for thiamine but the degree of heterotrophy for this varies widely (Mikola, 1948). Thiamine is often reported as being essential to the production of fungal fruiting bodies (Barnett and Lilly, 1947; Peterson, 1960). Robbins (1952) surveyed the growth requirements of some basidiomycetes (mainly saprophytes) concluding that a detailed study of vitamin utilisation was needed. Nutritional studies of a low temperature, parasitic basidiomycete by Ward (1962) indicated a complete requirement for thiamine and growth only resumed if the two thiamine moieties, thiazole and pyrimidine, were added together. Deficiencies in synthetic pathways of both the components are thus indicated. Norkrans (1950) reported that some *Tricholoma* species also had partial requirements for pantothenic acid and nicotinic acid. Biotin (Mikola, 1948) and inositol (Melin and Lindeberg, 1939) are also known to be deficient in various mycorrhizal fungi. Modess (1941) failed to obtain growth of several assumed mycorrhizal Hymenomycetes in pure culture and it was suggested that this may be partially due to complex vitamin requirements. Melin's (1962) "M-factor" possibly includes many vitamin complexes, in addition to carbohydrates, organic acids and auxins and nucleic acid constituents have also been implicated as growth stimulating substances (Melin, 1959; Nilsson - cited in Melin, 1963). Many of these micro-requirements are also

important in the germination of basidiospores (see Section 2.6).

2.3.4.2 Carbohydrates

The importance of carbohydrate sources to the fungal symbiont was first suggested by Frank (1885) and Melin (1925) confirmed this by showing that simple carbon sources such as glucose were the best growth promoters. Further work by How (1940 - B. elegans), Mikola (1948 - Cenococcum graniforme), Norkrans (1950 - Tricholoma species) and Melin (1963 - Amanita spp., Cortinarius spp.) emphasised the utilisation of simple carbohydrates produced photosynthetically by the hosts. Rommell (1938) showed that roots severed from the host, although mycorrhizal, did not allow production of fungal sporophores, concluding that this was due to the lack of carbohydrates normally produced by photosynthesis and translocated to the fungus. Some mycorrhizal fungi are capable of obtaining carbohydrates by litter decomposition. These have been shown to produce large amounts of extracellular polyphenol oxidase for the breakdown of lignin (Lindeberg, 1948). B. subtomentosus and L. deliciosus both produce the enzyme vigorously and it is significant that Rommell (1939) found sporophores of the former in areas where he had severed the roots from the host. Hacskaylo and Bruchet (1972) have also shown that certain species of Hebeloma can fruit in the absence of mycorrhizal roots. Melin (1946) indicated that B. variegatus could utilise forest litter if glucose was added and Norkrans (1949) found that T. vaccinum could utilise cellulose if glucose was present. Lyr (1963) found cellulolytic enzymes in some mycorrhizal

fungi: B. subtomentosus, B. luteus, B. variegatus and Amanita citrina, although B. badius and A. muscaria had no activity. All could attack hemicellulose. Foster and Marks (1967) considered that the cellulases produced by mycorrhizal fungi are inhibited by specific polyphenolic tannins secreted by the host, but the pectinases functional in Hartig net hyphal development during mycorrhizal synthesis are not. When penetrating the middle lamella of host root cells the necessary pectinases, hemicellulases and cellulases must be synthesised. Production of cellulase is inducible (Mandels and Reese, 1965) and it has been suggested (Melin, 1953; Norkrans, 1950) that the necessary enzymes are produced when available root glucose is diminished. Harley (1969) has also suggested that the fungus may interfere with the normal synthesis of cell wall material during the development of the cells of the epidermis - cap complex of the root, thus utilising the simple precursors of cellulose. The role of these enzymes is thus important to both root invasion and litter decomposition.

Adequate rates of photosynthesis must be maintained for mycorrhizal development. Formation of mycorrhizas in seedlings is retarded until the first foliage leaves have expanded (Robertson, 1954; Laiho and Mikola, 1964) and adequate light is available (Bjorkman, 1942; Harley and Waid, 1955). The concentration of soluble sugars in short roots increases with the frequency of ectomycorrhizas on the root system and Meyer (1974) considers this may be stimulated by fungal auxin production. Most mycorrhizal fungi produce their sporophores in late summer/autumn (compared with those of saprophytes, consistently present) and this may be caused by

competition with the host for carbohydrates during spring/ summer. Until active growth of the host slows and its auxin levels drop towards the end of its growing season, the fungus cannot compete for the carbohydrates and thus form sporophores (Meyer, 1968). The carbohydrates obtained from the host are, partially at least, converted into insoluble forms (glycogen, mannitol and trehalose) that are no longer available to the plant (Lewis and Harley, 1965). Ling-Lee et al. (1977a) found starch to be lacking in most cells of eucalypt mycorrhizas, although amyloplasts were noted in one sample. Glycogen was detected in fungal hyphae of the inner sheath region. The translocation and transformation of carbon compounds, with their effect on respiration of the mycorrhizas, are complex but have been reviewed by Hacskaylo (1973) and Harley (1969).

2.3.4.3 Mineral Nutrition

Mycorrhizas are important to the host to maintain supplies of phosphorus (P) and nitrogen (N) particularly where growing conditions are marginal and available levels of these and other trace elements low. The presence of mycorrhizas increase seedling dry weight and the efficiency of uptake of P, N and other minerals (Hatch, 1937). Plant response varies depending on the fungus and on available levels of the mineral in the soil. The response to mycorrhizal inoculation falls as mineral levels are raised (Bowen and Theodorou, 1967). Growth responses to various mycorrhizal fungi have also been shown by Moser (1956), Hacskaylo and Vozzo (1967) and Theodorou and Bowen (1970). Slankis (1967) indicated that the mycorrhizal system may exist

in a state of balance with host short roots retaining the ability to renew elongation when nutritional conditions for the host improve. N levels, when increased to concentrations at which formation of symbiosis is inhibited (Bjorkman, 1942) induced renewed growth in ectotrophic mycorrhizas of white pine (P. strobus). The host can therefore still exert some control over the symbiont.

In ectomycorrhizal roots, the uptake of minerals is largely determined by the symbiont since these enter the host through the fungal sheath, which also increases the effective root volume in the soil. Ionic uptake across the cell wall and plasmalemma has been reviewed by Harley (1969) and Bowen (1973). Recently Marx et al. (1977) have shown that formation of ectomycorrhizas in soils of varying fertility may be mediated through carbohydrate metabolism. Where levels of soil N and P were high, sucrose levels in loblolly pine (P. taeda) seedlings decreased, adversely affecting their susceptibility to ectomycorrhizal development. Powells and Krause (1959) found that at levels of N above 50 ppm, loblolly pine short roots were long, fleshy and had root hairs. At low levels (less than 5 ppm) distinct dichotomous branching of roots occurred. P levels had a similar effect, 1 ppm being the upper limit for dichotomy. Moser (1959) observed that auxin production by cultured mycorrhizal fungi was diminished when high concentrations of N containing compounds were available. At high N levels mycotrophic pine seedlings undergo reverse morphogenisis and symbiotic associations eventually terminated. The N metabolism of ectomycorrhizas has been investigated by Krupa and Branstrom (1974) and Krupa et al. (1973a). In the symbiotic system between B. variegatus

and P. sylvestris, amino acid fluctuations in mycorrhizal roots were found. Arginine, the major amino acid constituent of the fungus mycelium, was released from a bound form until about 70 percent was in a free pool in the hyphae during exponential growth. Bound forms of glutamic acid and glutamine, aspartic acid and asparagine and alanine also increased with time in different proportions suggesting production of diverse proteins. Arginine was again found to be a constituent of the uninfected root system, but with mycorrhizal initiation its free form decreased in concentration as compared to non-mycorrhizal roots. Other free amino acids, especially aspartic and glutamic acids, increased. It was suggested arginine availability in the host has a repressive effect on synthesis of the same in the mycorrhizal fungus, which utilises the host arginine pool instead. Any deficiencies in supply of N may therefore have important effects on protein synthesis in the mycorrhizal system.

Hatch (1937) indicated that mycorrhizas have a greatly enhanced uptake of P and the significance of mycorrhizal fungi with respect to P uptake has been investigated by many workers (Rosendahl, 1943; Melin and Nilsson, 1950; Purnell, 1958; Fowells and Krause, 1959, Ritter and Lyr, 1963; Bowen and Theodorou, 1967; Bowen, 1968; Mejstrik and Beneke, 1969; Bjorkman, 1970a; Mejstrik and Krause, 1973; Mejstrik, 1975) and reviewed by Harley and Lewis (1969), Bjorkman (1970), Voight (1971), Bowen (1973) and Slankis (1974).

The main site of P uptake occurs in the region of cell elongation behind the root apex and this is enhanced in the presence of mycorrhizas (Bowen and Theodorou, 1967).

It was also shown that pine mycorrhizas have a total absorbing power 2 - 9 times that of uninfected short roots. A similar situation is found with beech mycorrhizas (Harley and McCready, 1950, 1952). This work also indicated that uptake is much greater in the fungal sheath than in the host/ Hartig net 'core'. P uptake efficiency of mycorrhizal fungi varies and this may reflect their mechanisms of absorption. The presence of surface phosphatases on pine mycorrhizas has been reported by Paterson and Bowen (1968 - cited in Bowen, 1973). Phytase activity has also been reported by Theodorou (1968) for several fungi including S. luteus, therefore organic forms of P are available to mycorrhizas.

The presence of mycorrhizas increases the root volume in soil available for mineral uptake. Individual hyphae greatly increase the surface area and the presence of mycelial strands or rhizomorphs (Bowen, 1968) to exploit the inter-root soil allows efficient use of the available minerals which are then translocated via the hyphae to the host. Mycorrhizal efficiency is also seen by the replacement of root hairs in most symbiont situations by fungal hyphae. Skinner and Bowen (1974) have shown the effectiveness of mycelial strands in translocating 30-80 percent of absorbed P up to 12 cm in a field situation and Bowen (1968) suggested that those fungi producing such strands readily should be selected for inoculation purposes.

The fate of the P once it reaches the mycorrhizal sheath is still a point of conjecture. It is apparent that there is a pool of inorganic orthophosphate situated in the sheath (Harley and Brierly, 1954, 1955) and that the proportion of P passing to the shoot is smaller in mycorrhizal

seedlings. Morrison (1962) placed seedlings in a P-free medium after a period of uptake and found that although P was transported to the shoots of mycorrhizal and non-mycorrhizal plants in the former it continued for several days but fell off rapidly in non-mycorrhizal plants. Much work has recently been done to discover the form of this P-reserve (Harley and Loughman, 1963) and it is now suggested it may be stored as polyphosphate (oxygen linked tetrahedral PO_4 groups). Polyphosphates were first identified in living organisms by Schmidt *et al.* (1946) and Wiame (1947) and have since been found in many micro-organisms (Langen and Liss, 1958, Harold, 1966) as well as higher plants. Kulaev *et al.* (1960, 1975) have indicated polyphosphate presence in development of *Acetabularia* sporophores, as have Ebel *et al.* (1958) in *Saccharomyces* cells and *Aspergillus* mycelium. Jeffrey (1964) found long chain, high molecular weight polyphosphates in *Banksia ornata*, a heath plant capable of surviving in soils with low P levels, although Nassery (1969) could find no relationship between quantities of polyphosphate formed by *Deschampsia flexuosa* and *Urtica dioica* and their differences in growth response to P. Fernandez-Gomez *et al.* (1973) reported cytoplasmic inclusions of insoluble P in the roots of *Allium cepa* and Ashford *et al.* (1975) have found polyphosphate in the mycorrhizas of *Eucalyptus fastigata*. Metachromatic granules were detected within the Hartig net and inner sheath hyphae and it is suggested that these may be the form in which phosphate is bound in the fungus during periods of active P uptake. It can then be released as needed to the host. The formation of polyphosphates in *P. mugo* mycorrhizas is discussed in Chapter IX.

2.4 Pinus mugo (TURRA) GIOR. ITAL. (GRISILINI)

The Swiss or Dwarf mountain pine is also known as P. montana (Mill.) or P. echinata (Hort.) (Dallimore and Jackson, 1966). It is commonly found at high elevations in the mountains of central and southern Europe (the Alps, Pyrenees, Carpathians and Balkans). Some trees are found in Germany, Czechoslovakia and the USSR but are scattered. The growth form is variable, ranging from a small tree to dwarf bush-like growth. It has been found as high as 1,950 metres above sea level but is more common from 1,000-1,500 metres (Mirov, 1967). P. mugo is often closely associated with P. sylvestris in many parts of its natural range and it is thought that they may hybridise in nature. Three varieties are commonly found: var. mughus (Scopoli) Zenari, var. ucinata Ramond and var. pumilo (Haenke) Zenari. P. mugo can be recognised by its characteristic leaf structure. The needles are rigid, curved and blunt, arranged in pairs and persist up to five years. They are 3.5-8 cm long with a finely toothed margin and a basal sheath up to 1 cm in length.

According to Trappe (1962) P. mugo forms mycorrhizas with numerous basidiomycetes. B. edulis, S. subluteus and S. tomentosus are named as forming associations in pure culture and S. flavidus, S. granulatus, S. variegatus and S. luteus are other known symbionts among the Boletaceae. This work has shown that S. luteus may be included with the pure culture associates. Interest in the use of P. mugo for protection forestry in New Zealand dates back to the Mountain Land Forestry Symposium held by the Forest Research Institute

in 1968. From this it was evident that direct seeding and mycorrhizal trials needed more investigation. Trials carried out by the Forest Service's Wellington Conservancy in the Kaweka forest indicated that direct sown seeds, coated with mycorrhizal soil or duff, showed promise for reafforestation (Mountain Land Forestry Symposium, V.II, appendix 5, 6). P. mugo and Alnus viridus appeared to be the only two species practical for revegetation work and it was suggested that provenance trials be set up to test intraspecific growth variation of seedlings in their first two growing seasons under high country conditions (Beneke, 1968, Vol. II, pp 91). P. mugo shows resistance to all forms of physical damage and has vigorous growth which is reduced at the highest altitudes. It is very hardy in screes and var. Ucinata, in particular, is most resistant to windblasting.

Ledgard (1974) described the results of Forest Service direct seeding trials at altitudes above 1,000 metres. He found that P. mugo seedlings established best where competition with the primary cover of fescue/tussock was low, but where some protective cover by grasses was provided. On bare subsoil and screes seedlings did not survive the temperature extremes and frost heave during winter. Fertiliser addition trials showed maximum response when nitrogen and phosphate were added at the 50 kg/ha level, but unfertilised mycorrhizal seedling dry weight was still 32 percent greater than in these plants. The coating of seed with spores collected from sporophores under experimental tree plots did not prove successful in attempts to obtain good mycorrhizal formation. Field survival of seeds was only 0.4 percent after 13 months

from trial sites in the Craigieburn Range, the mortality being due to the harsh climate and animal predation. Good seed lines with high germination percentages must therefore be used. Self sown seedlings from established trees were not found (see also Ledgard, 1976).

Studies are now being carried out to monitor transpiration and CO_2 gas exchange of P. mugo and other species at high altitudes (Beneke et al. 1974, 1976). Results show that transpiration increases in the order: mountain pine/lodgepole pine/beech/larch and maximum CO_2 assimilation rates: mountain pine/beech/lodgepole pine/larch. The fact that mountain pine is the most drought resistant of these tree species is indicated by its high daytime xylem water potential and minimal alteration of its stomatal apertures.

Mountain pine is thus a very hardy tree under high altitude conditions; it can be direct seeded and most importantly forms ectotrophic mycorrhizas. Since ectomycorrhizal trees generally form the upper timberlines of mountain slopes (Moser, 1967) this means it has good potential for the afforestation of exposed, open sites.

2.5 Suillus (Boletus) luteus (L. ex Fries) S. F. GRAY

The Boletes, because of the tubular structures in which their spores are borne, were originally classified among the polypores by Fries (cited in Pearson, 1946). They also show affinities to the Agarics and several intermediate

forms are found. Paxillus paradoxus has gills joined by veins into tube-like structures and most other species of Paxillus have gills which partially anastomose and separate easily from the pileus, as in Boletes. There are, however, differences in spore shapes and colours and other physical dissimilarities which led to the formation of the order Boletales.

The most comprehensive classification of the family Boletaceae was that of Singer (1945, 1947, 1962). Singer recognised 14 genera in the Boletaceae and this taxonomic system is now widely accepted. A large proportion of species in this family are capable of forming ectotrophic mycorrhizas with forest trees and identification of these is described by Trappe (1962, 1967) and Dominik (1969). Singer and Kuthan (1976) have studied the sub-alpine and lower alpine zone of the High Tatras (Czechoslovakia) and have confirmed the P. mugho/S. luteus ectomycorrhizal association. In New Zealand species of Boletaceae appear to have been unintentionally introduced with their phanerogam partners and are obligatory mycorrhizal associates. These associations have been described by Rawlings (1951, 1958) and a full taxonomic description of the New Zealand Boletes is given by McNabb (1968). It appears that these members of the Basidiomycetes have become specialised, evolving from the saprophytic state to what is now a balanced symbiotic state (Hacskaylo, 1971). Further attempts at classifying the members of the Boletaceae will rely on such methods as chemotaxic analysis (Benedict, 1967; Catalfomo and Trappe, 1970; Harrison and Grund, 1975) and S.E.M. studies of spore types (Moore and Grand, 1970).

S. luteus is the type species for the genus Suillus and, like many mycorrhizal fungi, has been grown in pure culture (Pantidou and Groves, 1966) but is not known to produce fruiting bodies under these conditions. There have been few reports of sporophore production by members of the Boletaceae in vitro and little is known of the conditions required for their development. Pantidou (1961, 1962) first reported the production of Bolete sporophores in pure culture with Phlebopus species and McLaughlin (1964) was first to observe this with a member of the genus Suillus, S. rubinellus, grown on Hagem's medium. Pantidou (1964) also obtained sporophores of Xerocomus species but the medium was not given. The nutritional status of the fungus appears to be very important to the production of sporophores (Volz and Beneke, 1968; Hacskaylo, 1973a) and lighting conditions are also implicated (McLaughlin, 1964; Horikoshi et al. 1974; Kitamoto et al. 1974; Trembach, 1974). Matthews and Niederpruem (1972) have shown micrographs of sporophore primordium development in Coprinus lagopus and Uno and Ishikawa (1973) indicated the participation of adenosine 3', 5' - cyclic monophosphate in fruiting body induction of another Coprinus species. Recently Davis and Jong (1976) obtained fruiting bodies of Laccaria laccata on a rabbit food agar and Ashton (1976) has observed fruiting bodies from two Cortinarius species. Again light was important in their normal development. A Paxillus species, grown on a malt extract medium, was observed to produce sporophores under alternating light/dark light periods in this laboratory, but the culture has since lost this ability.

The production of fruiting bodies in pure culture is important as many basidiomycetes are otherwise unable to be distinguished. It also contributes to knowledge of their nutritional requirements. Although conditions are far removed from those in forest situations, the effects of individual factors can be studied. Cultures of S. luteus were grown on various media over a period of 8-12 months during the course of this thesis, but no sporophores were obtained. It appears that S. luteus still requires the presence of its host before fruiting will occur.

2.6 BASIDIOSPORE STUDIES

The development of inoculation procedures for introducing selected mycorrhizal fungi into tree planting sites has been determined by the types of inoculum available. These are either pure cultural fungal mycelium grown in bulk, mycorrhizal 'duff' from under existing forests, or basidiospores collected from sporophores. There are also problems such as inoculum failure under field conditions, deterioration under extended storage periods and insufficient supplies of the inoculum source to be overcome (Table 2.1).

Previous work has shown pure cultured mycelial inocula to be successful (Moser, 1958, 1958a, 1959; Gobl, 1975) and if growing conditions are stable or can be controlled, as in the nursery or growth room, this method is effective (Table 2.1C). Success of such inoculations under severe conditions, however, has not been evaluated, but deterioration of inoculum in the field and the vast quantities needed limit

TABLE 2.1 RECENT MYCORRHIZAL INOCULATION PUBLICATIONS

A DUFF/PINE LITTER OR MYCORRHIZAL ROOT INOCULUM

Host	Fungus	Author(s)
Pinus insularis	?	Thoen (1974)
P. oocarpa	?	Thoen (1974)
P. michoacarpa	?	Thoen (1974)
P. mugo	probably S. luteus	Ledgard (1974)
P. elliottii	?	Shoulders (1972)

B BASIDIOSPORE INOCULUM

Host	Fungus	Author(s)
Pinus radiata	Rhizopogon luteolus	Bowen et al. (1971) Theodorou (1971) Theodorou and Bowen (1973) Theodorou and Skinner (1976)
	Rhizopogon roseolus	Lamb and Richards (1974)
	S. granulatus	Lamb and Richards (1974)
	Pisolithus tinctorius	Lamb and Richards (1974)
P. taeda	P. tinctorius	Marx and Bryan (1975) Marx (1976) Marx et al. (1976)
	Thelephora terrestris	Marx and Ross (1970)
P. mugo	S. luteus	Ledgard (1974)
P. virginiana	P. tinctorius	Marx et al. (1976)
P. strobus	P. tinctorius	Marx et al. (1976)
P. elliottii	R. roseolus	Lamb and Richards (1974)
	S. granulatus	Lamb and Richards (1974)
	P. tinctorius	Lamb and Richards (1974)
Pseudotsuga menziesii	Laccaria laccata	Stack et al. (1975)

C PURE CULTURED MYCELIAL INOCULUM

1 ASEPTIC SYNTHESIS

Host	Fungus	Author(s)
P. radiata	R. roseolus	Lamb and Richards (1970, 1971)
	S. granulatus	Lamb and Richards (1970, 1971)
	Rhizopogon spp.	Lamb and Richards (1970, 1971)
	Cenococcum graniforme	Lamb and Richards (1970, 1971)
P. sylvestris	Boletus variegatus	Lundeberg (1960)
		Krupa and Fries (1971)
	B. subtomentosus	Lundeberg (1960)
	B. scaber	Lundeberg (1960)
	Rhizopogon rubescens	Froidevaux and Amiet (1975)
	Paxillus involutus 'E' strain	Mikola (1970)
		Laiho (1965)
(excised roots)	Amanita rubescens	Fortin (1966)
P. cembra	S. placidus	Froidevaux and Amiet (1975)
	S. variegatus	Froidevaux and Amiet (1975)
P. mugo	B. edulis ssp. edulis	Froidevaux and Amiet (1975)
P. elliottii	R. roseolus	Lamb and Richards (1970, 1971)
	S. granulatus	Lamb and Richards (1970, 1971)
	Rhizopogon spp.	Lamb and Richards (1970, 1971)
	C. graniforme	Lamb and Richards (1970, 1971)
P. echinata	T. terrestris	Marx and Davey (1969)
P. resinosa	S. granulatus	Richard and Fortin (1975)
P. ponderosa	P. involutus 'E' strain	Mikola (1970)
		Laiho (1965)
P. strobus	P. involutus 'E' strain	Mikola (1970)
		Laiho (1965)
Picea abies	P. involutus 'E' strain	Mikola (1970)
		Laiho (1965)
P. engelmannii	P. involutus 'E' strain	Mikola (1970)
		Laiho (1965)
P. sitchensis	P. involutus 'E' strain	Mikola (1970)
		Laiho (1965)

Host	Fungus	Author(s)
Pseudotsuga menziesii	Hebeloma crustuliniforme	Trappe (1967a)
	S. subolivaceus	Trappe (1967a)
	R. colossus	Trappe (1967a)
	Astraeus pteridis	Trappe (1967a)
	P. involutus	Mikola (1970)
	'E' strain	Laiho (1965)
Tsuga heferophylla	P. involutus	Mikola (1970)
Eucalyptus gummifera	Pisolithus tinctorius	Mullette (1976)

2 SEMI-ASEPTIC SYNTHESIS

Host	Fungus	Author(s)
Pinus radiata	R. luteolus	Theodorou and Bowen (1969, 1970)
		Skinner and Bowen (1974)
	S. luteus	Theodorou and Bowen (1970)
		Mejstrik and Krause (1973)
	S. granulatus	Theodorou and Bowen (1970)
	C. graniforme	Mejstrik and Krause (1973)
	'E' strain	Laiho (1965)
P. sylvestris	S. luteus	Ritter and Lyr (1963)
		Shemakhanova (1967)
		Mejstrik (1975)
	B. variegatus	Krupa and Branstrom (1974)
	B. bovinus	Mejstrik (1975)
	A. muscaria	Ritter and Lyr (1963)
	Paxillus involutus	Shemakhanova (1967)
	'E' strain	Laiho (1965)
P. taeda	Pisolithus tinctorius	Marx et al. (1976, 1977)
		Berry and Marx (1976)
P. ponderosa	S. granulatus	Riffle (1973)
	A. muscaria	Riffle (1973)
	A. pantherina	Riffle (1973)
	L. deliciosus	Riffle (1973)
	'E' strain	Laiho (1965)
P. strobus	P. tinctorius	Marx et al. (1976)
	'E' strain	Laiho (1965)
P. monticola	'E' strain	Laiho (1965)
P. edulis	'E' strain	Laiho (1965)
P. virginiana	P. tinctorius	Marx et al. (1976)
Pinus spp	B. bovinus	Bjorkman (1970a)
	B. subtomentosus	Bjorkman (1970a)

Host	Fungus	Author(s)
P. echinata	P. tinctorius	Berry and Marx (1976)
P. ayacahuite, P. lerophylla, P. michoacana, P. pseudostrobus, P. rudis, P. teocote	C. graniforme, P. tinctorius, T. terrestris	Marx (1975)
Picea abies	B. luteus	Mejstrik (1975)
	B. bovinus 'E' strain	Laiho (1965)
P. engelmannii	'E' strain	Laiho (1965)
P. glauca	'E' strain	Laiho (1965)
P. pungens	'E' strain	Laiho (1965)
P. sitchensis	'E' strain	Laiho (1965)
Pseudotsuga menziesii	'E' strain	Laiho (1965)
Tsuga heterophylla	'E' strain	Laiho (1965)
Eucalyptus regnans	Cortinarius ochraceus	Ashton (1976)
	C. radicus	Ashton (1976)
	Hypholoma fasiculare	Ashton (1976)
	Psalliota xanthoderma	Ashton (1976)
	Mesophellia arenaria	Ashton (1976)
	Clitocybe australiana	Ashton (1976)

In addition, the following fungi have been prepared in pure culture for nursery inoculations by Gobl (1975):

S. plorans, S. bovinus, S. grevillei, S. sibiricus
A. muscaria
Boletinus cavipes
H. crustuliniforme
Laccaria spp.
Lactarius spp.
Cortinarius spp.
Rhizopogon spp.

its practicability.

Little prior work has been done to develop a basidiospore inoculum using S. luteus (Table 2.1B) and information is lacking on basidiospore preservation, germination and viability. These aspects are reviewed in the following sections.

2.6.1 Basidiospore Germination

Fruiting bodies of many species of Suillus can readily be found in forest systems. A good supply of basidiospores is therefore available, but their reluctance to germinate has not facilitated their use as mycorrhizal inoculum. Germination of spores in field conditions is assumed to occur and although mycorrhizal fungi colonize non-host roots (Theodorou and Bowen, 1971), Wilde (1954), and Bowen (1963) indicated mycorrhizal fungi were absent from grasslands not in close proximity to ectomycorrhizal forest trees. Lamb (1974) found that the number of mycorrhizal fungi was greatly reduced at 600 metres from mature forest and that only basidiospores spread the species at 1 kilometre. He considered that most dispersal was probably by vegetable mycelium or resting structures (chlamydospores or oidia). It appears that the spores may undergo a prolonged period of maturation or dormancy (possibly exogenous) after their release from the basidia (Cochrane, 1974).

Fries (1941, 1942) obtained cultures from spores,

including those of B. luteus and B. granulatus, on a malt extract medium which were used in mycorrhizal synthesis experiments. He found that the presence of certain yeast colonies activated germination of the spores. The activator yeast Torulopsis sanguinea gave less than 0.1 percent spore germination, but Fries postulated that the effect was due to either inhibitor neutralisation or germination stimulation. Bulmer and Beneke (1961, 1962) found that Calvatia gigantea basidiospores gave germination of less than 0.001 percent after long periods of incubation. They also noted that only spores from certain sporophores germinate and that spore concentrations affected germination. An after-ripening period of 2-3 years was also indicated. The spores of Calvatia thus exhibit characteristics that are also evident in spores of many Boletes.

Stimulation of spore germination has been shown for some micro-organisms. Agaricus bisporus spores have been closely studied and a metabolite of the fungus (Losel, 1964) has been shown to stimulate spore germination (Losel, 1967, Rast and Stauble, 1970). In addition to isovaleric acid acting as a self-stimulator of germination, CO_2 acts as a self-inhibitor by repressing the action of the Krebs cycle enzyme succinate dehydrogenase (Rast et al. 1974, 1976). The mechanism of germination is therefore well known in Agaricus spores, but that of many other basidiomycetes is poorly understood. Basidiospores of Pholiota aurivella, a wood decaying fungus, germinated on a malt extract medium, reaching 30 percent after several days (LaVallee and Lortie, 1971) and spores of Schizophyllum commune have several known

nutritional requirements for germination (Niederpruem and Denner, 1966). Germination of spores belonging to members of the Hymenomycetous heartrot fungi is very erratic (Merrill, 1970; Brown and Merrill, 1973) but Watling (1963) observed germination in spores from herbarium material of species of the Bolbitiaceae after previously incubating these overnight in a saturated atmosphere. Again malt extract was used as the growth medium.

Fries (1966) has reviewed factors affecting basidiomycete spore germination and indicated that with many of the mycorrhiza forming Hymenomycetes and Gasteromycetes activator organisms, such as the yeast Rhodotorula mucilaginosa var sanguinea, are essential, particularly with the Boletes. Lamb and Richards (1971, cited in Bowen and Theodorou, 1973) found that an activator from R. glutinis also stimulated germination of spores from S. granulatus, R. roseolus and P. tinctorius. With some Boletus species, growing mycelium serves as an activator, but B. scaber and B. rufus spores have not responded to any activators. Occasional germinations occurred after long incubation periods (4-6 weeks) and the resulting mycelium often stimulated nearby spores to germinate. Fries also considered that an inhibitory substance was present in media containing extracts of malt or yeast, which the activator organism removed. Using spores of B. luteus he obtained up to 1 percent germination on a 'non-inhibitory' medium. However, if fresh spores or completely imbibed spores are used, germination readily occurs although the percentage may still be low (Watling, 1971).

It is evident, therefore, that many factors are involved in basidiospore germination but much study is still needed, particularly on the effects of stimulatory compounds such as furfural (Sussman, 1953; Peterson, 1960) and those known to be components of host root exudates (Bowen and Theodorou, 1973).

2.6.2 Basidiospore Preservation

An important problem in the development of a successful mycorrhizal inoculum using basidiospores is the survival of these spores during the necessary storage period. After collection in late summer, spores must be stored in such a manner that their viability is maintained until they are used at the time seeds can be sown. Mature spores released from basidia in the sporophore appear to be best as an inoculum source as preserved spore bearing hymenial tissue contains many immature basidiospores.

Lamb and Richards (1974) found that for spores of R. roseolus, P. tinctorius and S. granulatus, optimum germination occurred at temperatures of approximately 20°C, but 48 hour exposures of the spores to temperatures of 52°C, 56°C and 50°C respectively, in irradiated soil, were lethal. Relative humidity was also shown to have an effect on viability after spore storage of the above species. The optimum level was approximately 50 percent following 60 days storage, but the tolerance range, particularly of P. tinctorius spores, was very wide (from 10-99 percent).

Storage of spores at low temperature was successful in maintaining spore viability according to Fries (1943) although Moser (1958 b) indicated that some mycorrhizal fungal cultures can also survive temperatures of -11° to -12°C *in vitro*. Bulmer and Beneke (1962) obtained greatest spore viability with *Calvatia gigantea* when sporophores were stored at -18°C and low temperature storage has also been used by Marx and Ross (1970), LaVallee and Lortie (1971), Brown and Merrill (1973), Marx (1976) and Marx *et al*. (1976).

It is also possible to freeze dry spores for later use as an inoculum source. Theodorou and Bowen (1973) and Stack *et al*. (1975) have used this method successfully for prolonged storage.

These methods are promising for basidiospore preservation but methods of producing asexual spores or sclerotia (Laiho, 1970; Lamb and Richards, 1971) by mycorrhizal fungi *in vitro* may also have considerable potential.

2.6.3 Basidiospore Inoculation of Seeds

The important factors involved in mycorrhiza inoculation have been reviewed by Bowen (1965), Bowen *et al*. (1971) and Trappe (1977). Basidiospores have been used as a source of inoculum by adding them to soil or other plant growth media. Spores of *P. tinctorius* (Lamb and Richards, 1974; Marx, 1976; Marx *et al*. 1976; Mullette, 1976), *Laccaria laccata* (Stack *et al*. 1975), *R. roseolus* and

S. granulatus (Lamb and Richards, 1974) and Thelephora terrestris (Marx and Ross, 1970) proved successful in mycorrhizal formation.

Theodorou (1971) used spores of R. luteolus, deposited on the seed coat of P. radiata seeds, to effectively produce mycorrhizas. Greatest inoculation response was obtained after soil sterilisation which may have been due to elimination of competition by saprophytes and/or pathogens (Warcup, 1952; Theodorou, 1967; Ridge and Theodorou, 1972) or by releasing soil nutrients (Warcup, 1957). Further studies by Theodorou and Bowen (1973) showed that freeze dried spores were a good inoculum source and that spores on inoculated seeds could survive cold storage successfully before planting out. To give results equivalent to those with fresh spores, however, numbers of spores when treated by freeze drying and cool storing, had to be increased 100 fold and 10 fold respectively. Even at the highest concentrations of spores used (10^5 spores/pot) infection was still found to be increasing and also stimulation of basidiospore germination in the host root rhizosphere was observed. The influence of fungicides on mycorrhizal development was studied by Theodorou and Skinner (1976) who found that captan, zineb and thiram, commonly used to control seedling damping off, all inhibited formation of mycorrhizas on seeds inoculated with spores. Naturally occurring mycorrhizal fungi in the soils were, however, not affected and mycorrhizas developed normally. They also attempted to aid mycorrhizal development by placing a pellet containing spores below the fungicide dressed seed which

was successful but the effect of coating the seed with the pellet material was not investigated.

Little work has been done to develop an effective seed granulation technique and the study reported in Chapter VII is an attempt to increase knowledge of basidiospore inoculation techniques.

CHAPTER III

COLLECTION AND TAXONOMY OF S. luteus SPOROPHORES

3.1 INTRODUCTION

In the course of revegetation studies carried out by the Forest Service at Craigieburn, it was noted that the predominant sporophores present in forest tree plots were those of S. luteus (Ledgard, 1974). Its sporophores have also been found under P. radiata stands on dune soils adjacent to beaches of Pegasus Bay (Canterbury). In this situation, however, they are very sparse and the main species appear to be S. granulatus, S. brevipes and S. subacerbus. Sporophores of S. elegans, associated with Larix species, can also be found in the high altitude situation. S. luteus sporophores can withstand the harsh conditions found at altitudes above 1,000 metres, therefore it appears best suited to mycorrhizal inoculation in these high country areas and a collection programme was carried out through the 1975, 1976 and 1977 growing seasons. The sporophores were initially identified using the taxonomic data given by McNabb (1968).

3.2 MATERIALS AND METHODS

Mature sporophores had the stipe excised when

collected and were placed in boxes for transport to the laboratory. They were then placed on large glass sheets after being checked for insect infestation and cleaned of excessive litter and dirt. Most sporophores lasted 2-4 days before deteriorating and each morning the basidiospores released from the hymenium were scraped from the glass with razor blades and stored in dated bijou bottles. Spore samples were thus composed from 20-30 sporophores and no attempt was made to collect separate spore samples from individual sporophores.

Immature sporophores were collected and used to obtain pure cultures of S. luteus for experimental work discussed in Chapter IV. During identification studies, sporophore tissue reaction with the following chemicals was noted: 10 percent KOH, 10 percent NaOH, 10 percent NH_4OH and Conc NH_4OH. Sporophore tissue and spores for observation with a Cambridge Stereoscan 600 S.E.M. (at 15 Kv) were prepared by freeze drying and mounting with double sided celluloid tape on stubs. They were then vacuum coated with gold/palladium. Spores observed with a Hitachi HS-75 T.E.M. (at 50 Kv) were fixed in phosphate buffered glutaraldehyde (2.5 percent) at pH 7.0 and then postfixed overnight in buffered 1 percent osmium tetroxide (pH 7.0) at 4°C after being embedded in 2 percent molten agar and cut into 1 mm square blocks. They were washed and passed through a graded alcohol series before being placed in propylene oxide and embedded in Araldite. This was polymerized at 40°C for 24 hours and 60°C for 48 hours. Blocks were sectioned on an LKB ultrotome II using glass knives and the sections were

mounted on Formvar-coated copper grids and stained with 1.5 percent $KMnO_4$ (aqueous). Light microscopy was done using a Bausch and Lomb or Leitz Orthoplan microscope.

3.3 RESULTS AND DISCUSSION

The growth season of S. luteus sporophores starts in mid to late February and lasts until approximately midway through April in the Craigieburn area. Climatic data for this area is given in Table 3.1. Initiation of sporophore production appears to have no definite climatic correlation, although lowered temperatures and available moisture are important. Sporophores are often found within a week after rainfall. The end of their production is determined by increasingly colder temperatures during April, with ground frosts becoming common and occasional snowfalls being experienced. Actively growing sporophores were found even after snow had been lying on the ground for several days, but they did not reach sizes found earlier in the season. It is possible that initiation of the S. luteus fruiting season is determined by cessation of active tree growth in late summer-autumn as postulated by Meyer (1968). The fungal auxin levels then become greater than host produced auxin levels and sporophores are produced. This, however, is not consistent with the findings of Fortin (1967) who considered that the tree acts as a sink for fungal auxins which normally repress mycelial growth, unless the raised levels of auxin of fungal origin stimulate fruiting.

The physical characteristics of the sporophores are

TABLE 3.1 CLIMATE OBSERVATIONS IN THE CRAIGIEBURN RANGE

Craigieburn Forest site
(915 metres, N.W. aspect)

		J	F	M	A	M	J
Monthly Mean Air Temps. (°C)	1975	14.5	12.5	12.2	9.1	6.2	1.6
	1976	12.8	10.5	12.6	9.5	5.0	1.6
	1977	10.8	13.8	12.9	8.7	3.9	2.6
Mean		12.7	12.3	12.5	9.1	5.0	1.9

Monthly Mean Soil Temps. at 10 cm	1975	20.4	18.4	16.7	12.3	8.4	4.2
	1976	17.0	15.2	15.8	10.6	5.3	1.5
	1977	13.2	15.8	14.1	10.1	5.1	3.1
Mean		16.8	16.4	15.5	11.0	6.2	2.9

Precipitation Totals (mm)	1975	174.1	106.2	59.2	210.4	193.2	115.0
	1976	173.3	51.0	42.0	70.6	134.3	157.7
	1977	203.4	66.2	18.1	106.5	78.6	107.0
Mean		183.6	74.4	39.7	129.1	135.4	126.5

Ground Frosts per Month	1975	0	0	1	9	15	28
	1976	1	2	3	16	26	25
	1977	0	1	3	17	25	24

as follows (Fig. 3.1, 3.2). The pileus is generally convex in shape ranging from 5-10 cm in diameter, but numerous examples have been found exceeding 20 cm. Fig. 3.3 indicates the variable sizes of sporophore that may be found. The colour of the pileus is a dull red-brown to chocolate-brown and the cuticular layer is mucilaginous under damp conditions, being readily separable from the underlying tissue. The stipe is 4-9 cm long and 1-3 cm in diameter on which are found glandulae and a substantial annulus. The fruiting bodies may be recognised by their retention of an annulus. Fragments of the annulus are often left around the edges of the pileus as it expands, leaving a torn veil. In the maturing sporophore the annulus is typically white with a pink-purple colouration underneath. It remains attached to the stipe, often intercepting large numbers of spores. The glandulae are found apically on the stipe, which is a light yellow colour above the annulus and light brown below. When broken, the pileus has a white or pallid yellow interior which does not change on exposure to air. The reaction of KOH, NaOH and NH_4OH on the inner tissues of the pileus were similar. All gave immediate red-pink flushes of colour which then turned blue-violet. These reactions correspond to those obtained by Pantidou and Groves (1966) and McNabb (1968) for S. luteus.

The hymenial tissue consists of numerous pores (Fig. 3.4) 5-10 mm long and 0.5-1 mm in diameter, which are adnate or subdecurrent to the stipe. They are a pallid yellow colour when young and become deep ochre yellow at maturity. The hyphae forming the matrix of the hymenium are

FIGURE 3.1 S. luteus sporophore.

Top view of pileus. x0.5

FIGURE 3.2 S. luteus sporophore.

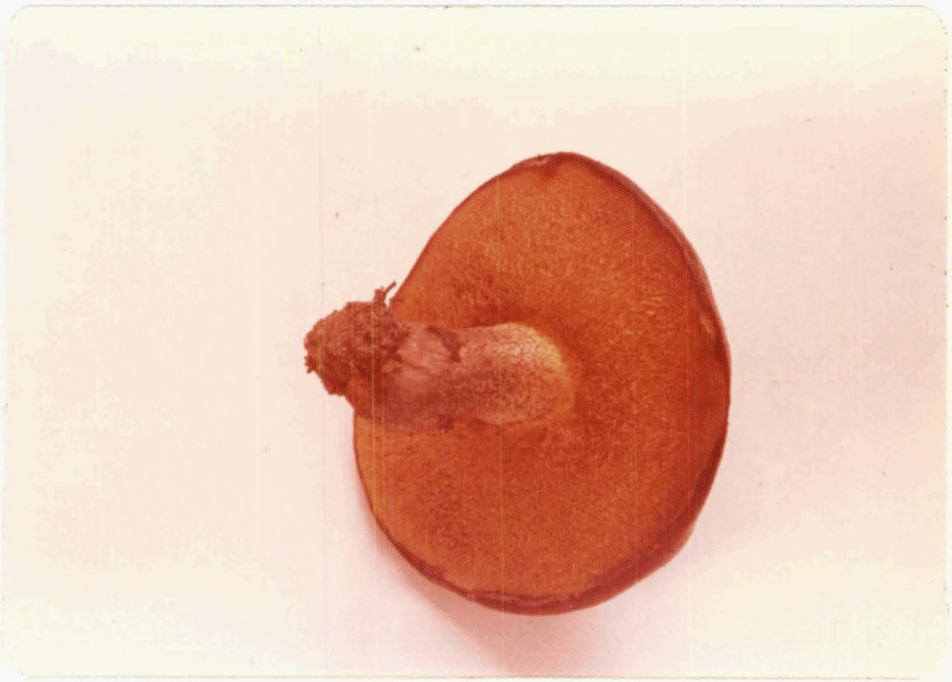
Bottom view of pileus. Note presence of annulus on stipe, the yellow-brown pores and remnants of the veil around the edge of the fruiting body. x0.5

FIGURE 3.3 Relative sizes of S. luteus sporophores.

Large fruiting body is about 25 cm in diameter. x0.125

3-1

3-2



3-3

FIGURE 3.4 Structure of S. luteus pore (scanning electron micrographs).

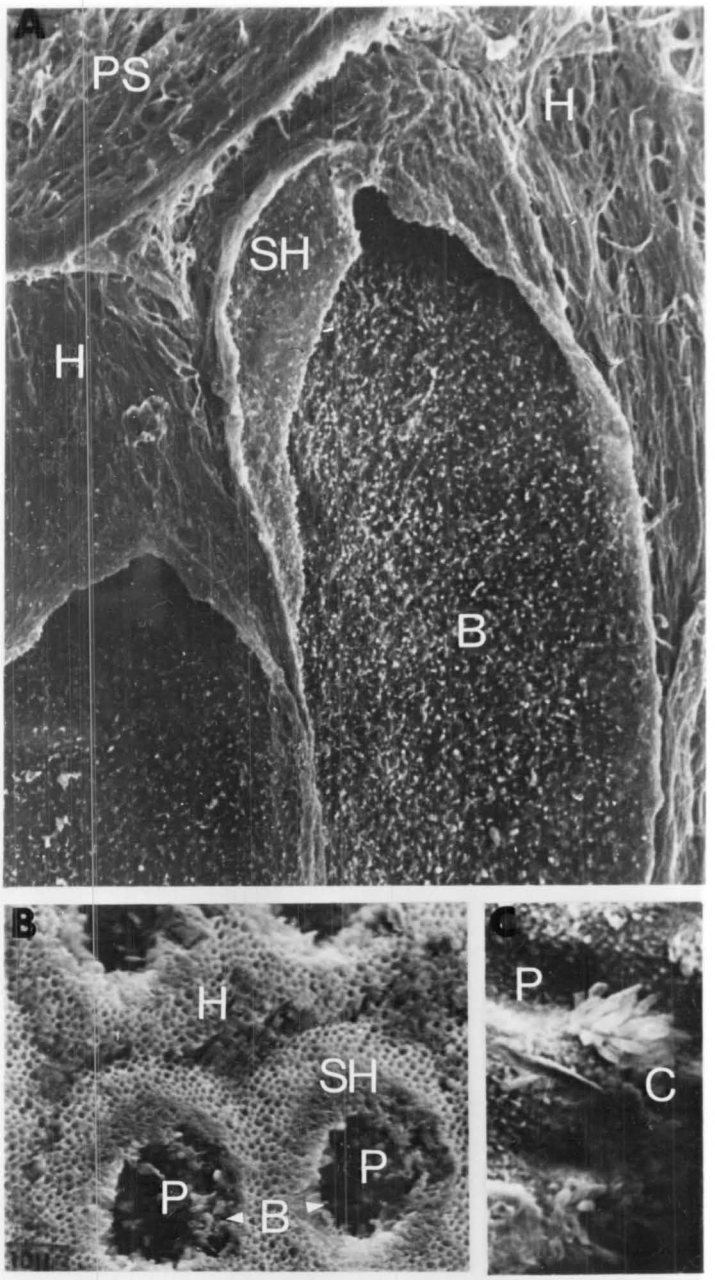
A L.S. section of pore showing hymenium (H) pileus (PS) sub-hymenial layer (SH) and numerous basidiospores (B) on inner surface. x50

B T.S. section of several pores (P) lined with basidiospores (B) showing matrix of hyphae forming the hymenium (H) and sub-hymenial layer (SH). x14

C Cystidia (C) found at the opening of each pore (P). x40

FIGURE 3.5 Basidiospores (B) scattered over inner surface of pore. x720

3-4



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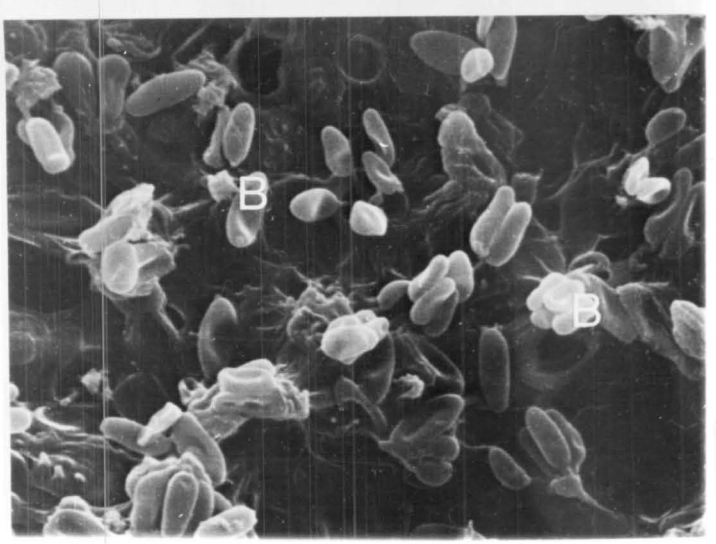


parallel and aligned on the vertical axis of the sporophore (Fig. 3.4 inset (A)) and numerous cystidia are found at the opening of the pore (Fig. 3.4 inset (C)). Numerous basidiospores are evident within the pores (Fig. 3.5) and are borne on the basidia which have four sterigmata (Fig. 3.6). The mature spores range in size from 7.5-12 µm in length and 4-7.5 µm in width (Fig. 3.7) and leave a yellow/brown spore print. No ornamentation of the spores was seen except for the conspicuous hilar appendage where the spore attaches to the sterigma. In a study of spore ornamentation, Moore and Grand (1970) found that many Boletaceae spores are smooth although those of Phylloporus rhodoxanthus are roughened. Those of Lentinus edodes also show a rough surface (Nakai and Ushiyama, 1974) which the authors consider are an artifact. The ultrastructure of sterigma growth and basidiospore formation in B. rubinellus was shown by McLaughlin (1973). Preliminary T.E.M. studies in this laboratory indicated a very thick spore wall. Few interior details could be seen because the sections were cut with a glass knife and the hard spore coat did not allow sufficiently thin sections to be prepared. A spore section is shown in Fig. 3.8, in which the very thick spore wall is evident. The cytoplasm fills a large proportion of the spore lumen and is thought to consist mainly of lipids. The remaining cytoplamic material may be similar to that reported by Greuter and Rast (1975) and Wells (1965) in Agaricus and Schizophyllum spores which consist of a matrix containing glycogen granules and free ribosomes (See Chapter V).

Problems of collection of sporophores and subsequent

FIGURE 3.6 Basidiospores (B) attached by their sterigmata (S) to a basidium (BM). x3900

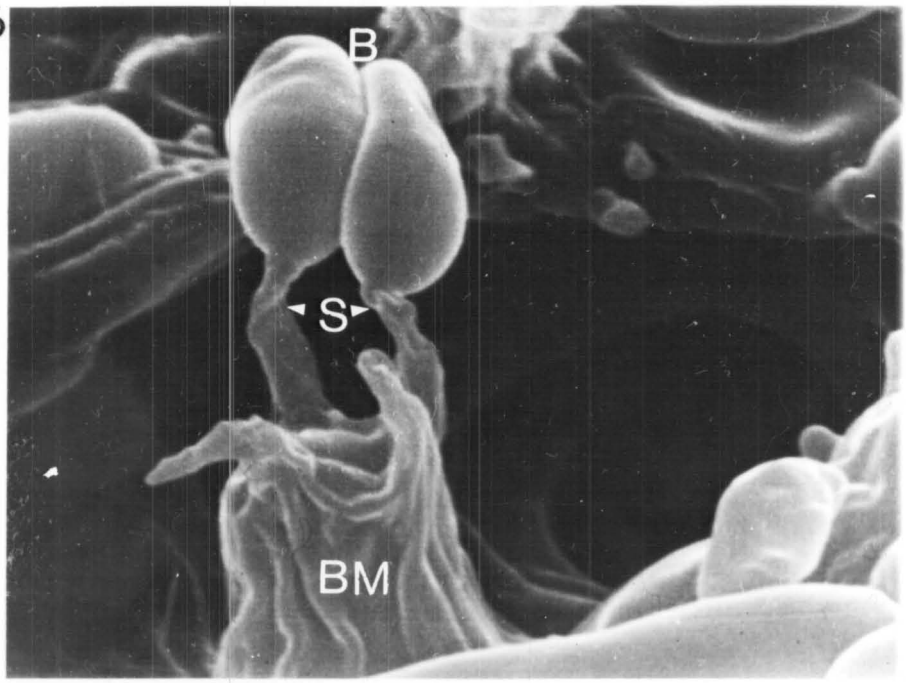
FIGURE 3.7 Structure of the basidiospore.

A Scanning electron micrograph showing smooth surfaced spore. Note the attachment point of the sterigmata (AP). x3750

B Light micrograph showing division of spore cytoplasm into vacuolate-like structures. (V). x1800

C Light micrograph showing thick spore walls (W). x1860

3-6



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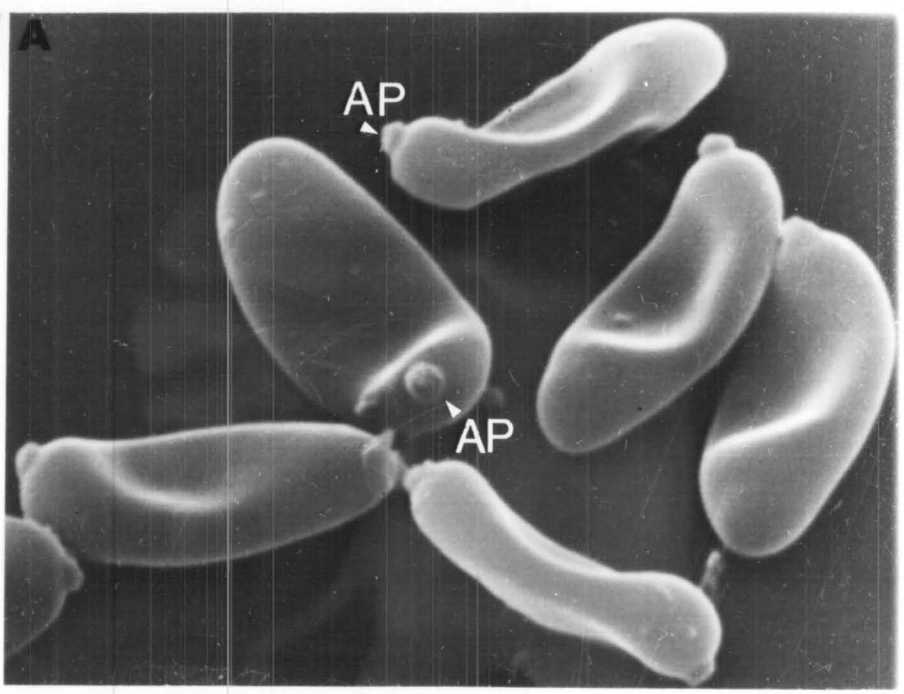
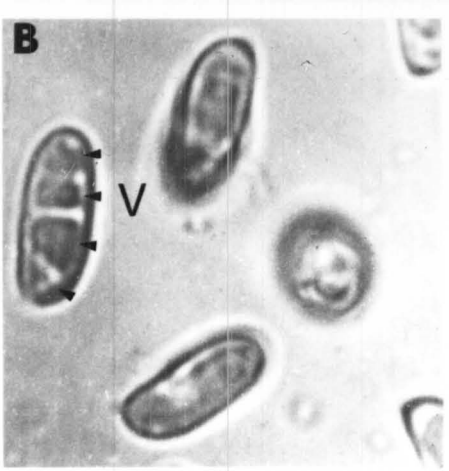




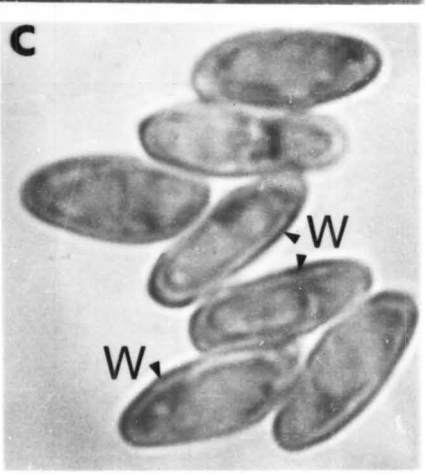
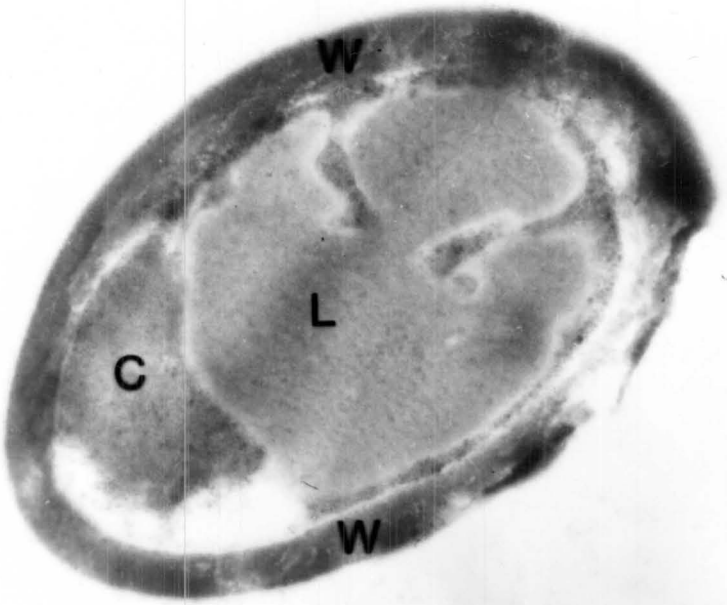


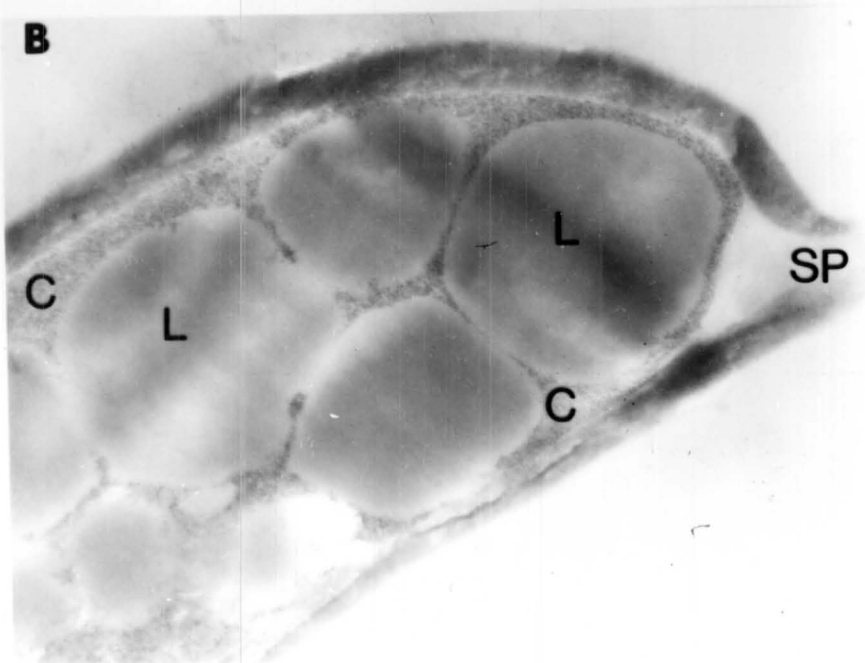
FIGURE 3.8 Basidiospore ultrastructure (transmission electron micrographs).

A T.S. section showing large lipid body (L) in cytoplasmic matrix (C). Thick cell wall (W) is also evident. x15,000

B L.S. section of basal portion of spore showing spore plug (SP), originally attached to a sterigma, and globular lipid bodies (L) in granular cytoplasmic matrix (C). x15,000

3-8 A





storage periods for the spores may eventually be overcome by their being raised in pure cultures, although so far S. luteus fruiting bodies have not been produced in this manner. Further studies on the conditions required for sporophore initiation are needed. Little is known of the factors affecting germination of basidiospores - the presence of the thick spore wall may prevent entry of water and exogenous substances necessary to begin this process, as well as protecting the spore - and methods of determining their viability need to be found.

CHAPTER IV

PURE CULTURE STUDIES OF S. luteus

4.1 INTRODUCTION

The importance of S. luteus as a mycorrhizal symbiont has stimulated a number of studies on its physiology and factors affecting its growth (Melin, 1954, 1959; Hacskaylo et al. 1965). Isolates of this symbiont were made during the present study from sporophore tissue and spores. These were then investigated for their growth characteristics with, and utilisation of, various carbohydrates, organic acids, nitrogen sources and phosphate and pH levels. Pure cultures were also used to facilitate identification of the fungus and the effects of growth on a number of commonly used laboratory media were noted.

4.2 MATERIALS AND METHODS

Media recommended for the culturing of mycorrhizal fungi are based on the ingredient Malt Extract (M.E.). Initially pure cultures of S. luteus were isolated on the M40 agar medium of Stevens (1974) but various other media were also tested. Compositions of the agar and broth media routinely used during cultural studies are given in Appendix 1.

4.2.1 Isolation of the Fungus

Pure cultures of S. luteus were isolated from young sporophores under aseptic conditions. The pileus was broken apart under sterile conditions in an Envirco ENV. 48 laminar flow cabinet (Biodynamics Inc.) and 1 cm^3 blocks were cut from the exposed hymenial tissue. Usually three or four blocks were placed on M40 or PDA plates (Appendix 1, media A, C) supplemented with thiamine and biotin. Stevens (1974) states that the fungal tissue should be partially embedded in the agar for growth to begin. On both these media, however, good growth occurred from the blocks and colony diameter increased by 2-3 mm/day under dark conditions at 25°C. Only young sporophore tissue was used, as that from older sporophores tends to autodigest rapidly when removed from the pileus. Once cultures were growing successfully they were maintained on M40 McCartney slopes at approximately 5°C and sub-culturing was carried out at periods of 2-3 months without noticeable loss of vigour. Cultures were also obtained from agar plates on which spores had germinated and from mycorrhizal roots surface sterilized with 10 vol. hydrogen peroxide for 1-2 hr, washed in sterile distilled water and plated on M40. Inocula for the pure culture studies were obtained by cutting small (5 mm x 5 mm) blocks from M40 A plates and adding these to sterile experimental flasks.

4.2.2 Pure Culture Studies

Growth of the fungus on agar surfaces was estimated

by measuring radial increases of the colonies. Static liquid cultures were used for nutritional requirement studies. A broth of medium G was used for the N source, P level and organic acid experiments and medium E for the carbohydrate experiment (see Appendix 1). The appropriate nutritional factors in these media were replaced by those being tested. All were carried out in 100 ml Erlenmeyer flasks containing 20 or 25 ml aliquots of growth medium. Cultures were harvested after one month's growth at 25°C in the dark and placed in pre-weighed aluminium cups, dried to constant weight (48 hours at 70°C) and reweighed. pH measurements were made using a Radiometer PHM 22r or Digi-Sense (Cole-Palmer) meter. Media were sterilised in a steam autoclave for 20 minutes at 122°C.

4.2.3 Statistical Analyses

All experiments showing clear differences in results between treatments were not analysed statistically. When results were analysed, either the students t-test, or an analysis of variance in conjunction with Duncan's new multiple range test, were used. In mycorrhizal synthesis experiments, the numbers of mycorrhizas per root ranged from approximately 1-40 percent and in such cases the data was firstly transformed, using an arcsin

transformation, before being analysed. In tables of experimental results, treatment means are designated alphabetical letters denoting their ranking (A equals highest order of ranking) and significance. Means having a letter in common are not significantly different. Significance at the one percent level (**) is given by capital letters and that at the five percent level (*) by lower case letters.

4.3 RESULTS AND DISCUSSION

4.3.1 Mycelial Characteristics

These correspond closely to those described by Pantidou and Groves (1966). The radial growth on M40 A agar was approximately 4 cm per month. In appearance the colony was furry or woolly with the centre a cinnamon pink colour becoming darker with age. The medium was often stained a yellow brown colour, the intensity being dependent on the medium composition. PDA gave intense colouration but agar media containing ammonium tartrate as the N source showed little. Brown exudate droplets were formed on the surface of the colony during rapid growth and were investigated by paper chromatography. They were shown to

contain a water soluble pigment composed of two distinct parts giving Rf values of 0.9 and 1.0 respectively when run on Whatman No. 1 chromatographic paper using a distilled water solvent (Smith, 1969). In non-staining media only the Rf 0.9 spot was detectable in the fluid drops. Both collidine and ninhydrin gave positive reactions for amino acids and alkaline $KMnO_4$ or $AgNO_3/KMnO_4$ gave positive reactions for reducing compounds (Smith, 1969). Similar exudates were noted on S. elegans cultures by How (1940). Liquid cultures became pigmented with certain media also (Fig. 4.1).

Aerial hyphae are numerous and range in diameter from 1.5-3 µm. These hyphae commonly exhibit simple and paarige (pairs of branches produced immediately below a transverse wall) branching (Fig. 4.2) and many hyphae have a papillated surface (Pantidou and Groves, 1966) which is also a distinctive feature seen in S.E.M. micrographs (Fig. 4.3). Clamp connections were not observed in cultures from sporophore tissue or from germinated spores. They were, however, seen on mycorrhizal roots formed in aseptic or semi-aseptic synthesis studies (Fig. 4.4). Melin (1923) described two groups of Boleti based on their mycelial characteristics:

A Mycelium with frequent paarige branching and numerous clamp connections.

B Mycelium with infrequent paarige branching and few clamp connections.

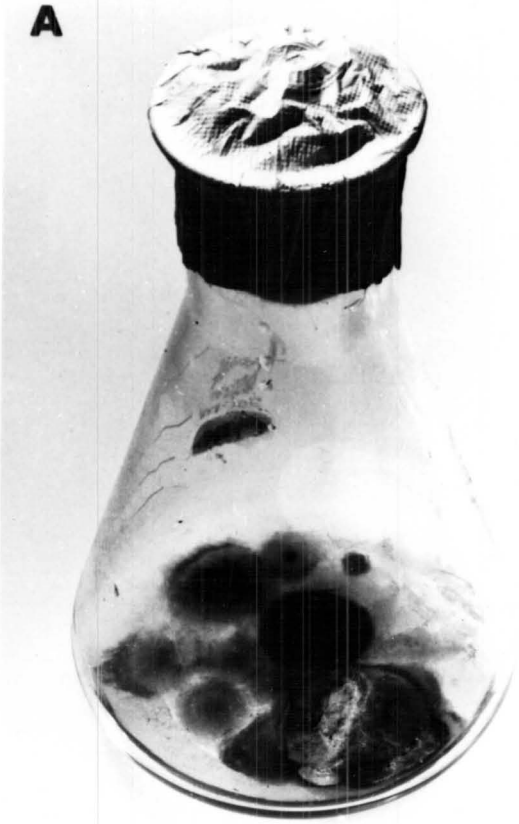
FIGURE 4.1 Pigmentation colour of S. luteus cultures in liquid culture.

A Growth of cultures in M40 A broth - extensive mycelial growth is evident and no pigmentation occurs.

B Growth of cultures in M.E. broth - mycelial growth is less extensive and culture medium becomes pigmented.

Both x0.75

4-1 A



B

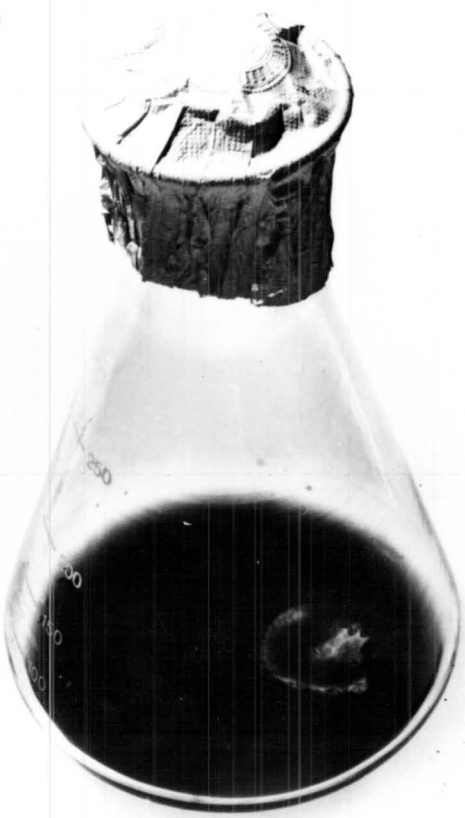


FIGURE 4.2 Hyphal branching (light micrographs).

A&B Examples of simple branching (arrow) occurring below a transverse hyphal wall (W). x350

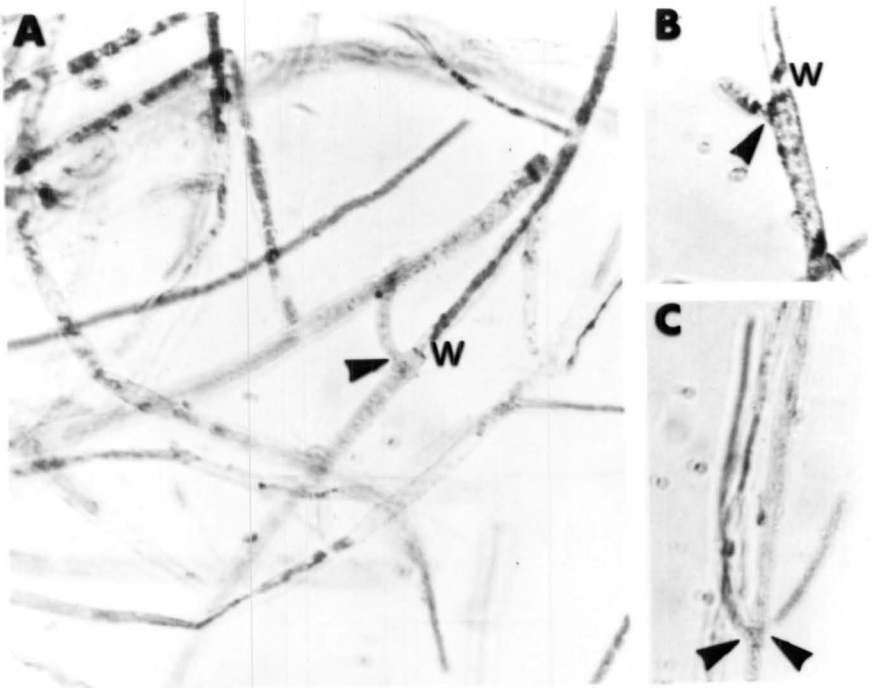
C Paarige branching (pairs of branches produced below a transverse wall. x320

FIGURE 4.3 Surface features of hyphae (scanning electron micrographs).

A Papillated hyphae.
P = papillae. x8200

B&C Hyphae apparently papillated but also with globular adcrusting substances (G) on the surface. x2200
x2700

4-2



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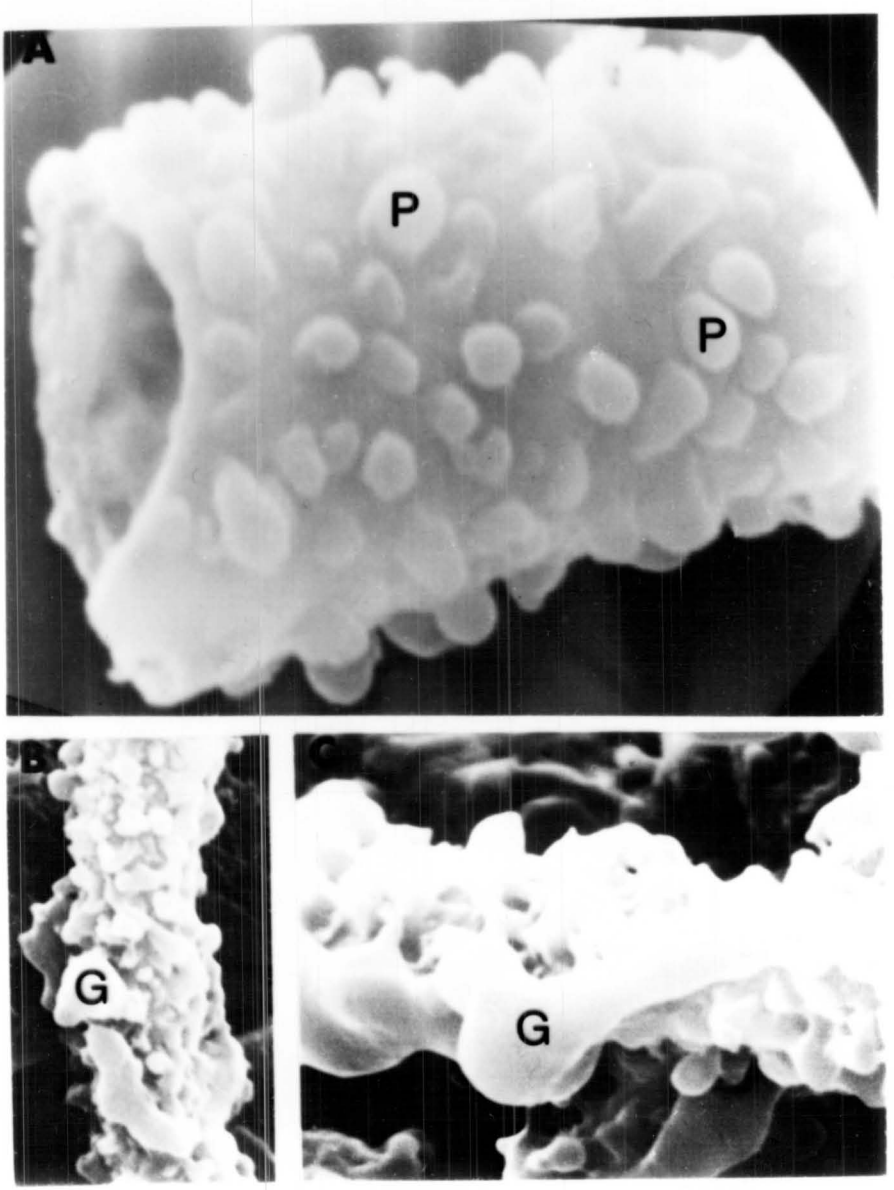


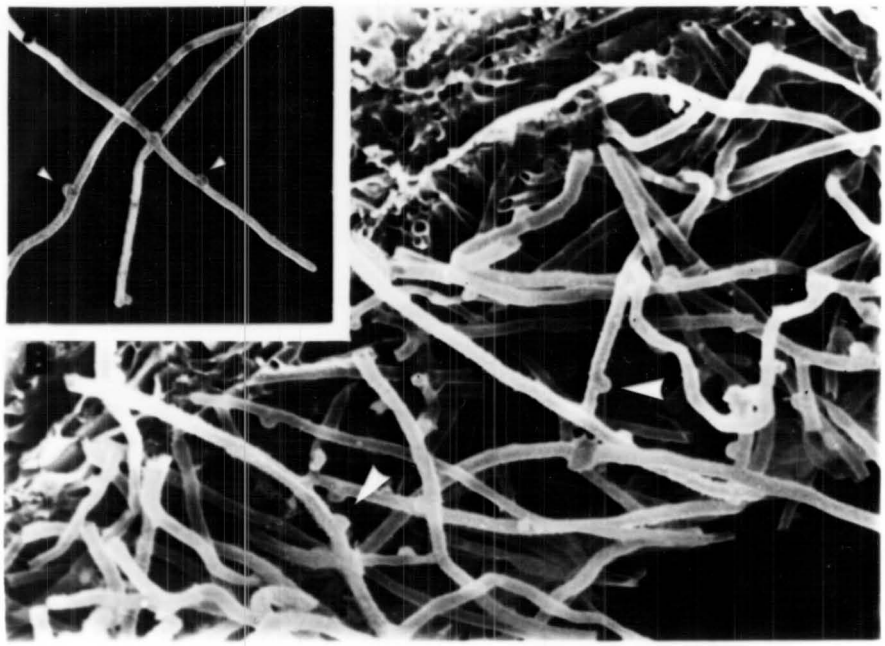
FIGURE 4.4 Clamp connections (scanning electron micrographs).

A In free hyphae (arrows). x170

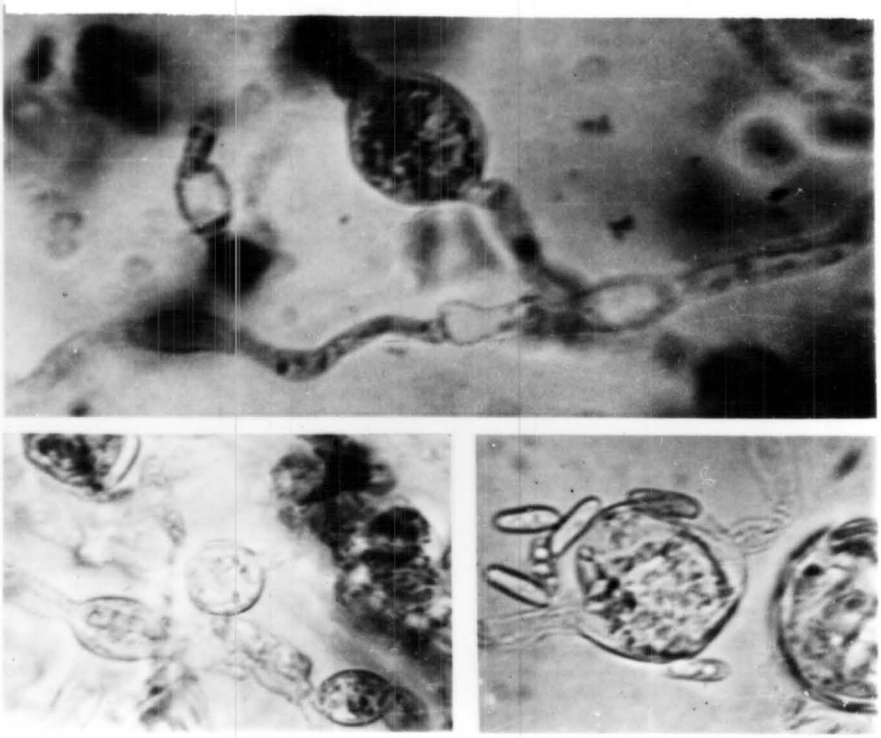
B In mycorrhizal sheath hyphae. x220

FIGURE 4.5 Sap hyphae - commonly found on the surface of, or embedded in, the nutrient agar gel. Note size in comparison to basidiospores. x450

4-4



4-5



He included B. luteus in the first group but cultural observations of the strain isolated during this study indicate features of both groups, ie paarige branching and infrequent clamp connections. Differing growth conditions must be considered when characterising the mycelial type exhibited. This would also agree with How's (1940) findings that there are greater similarities between mycelia of Boletus species than had been suggested by Melin.

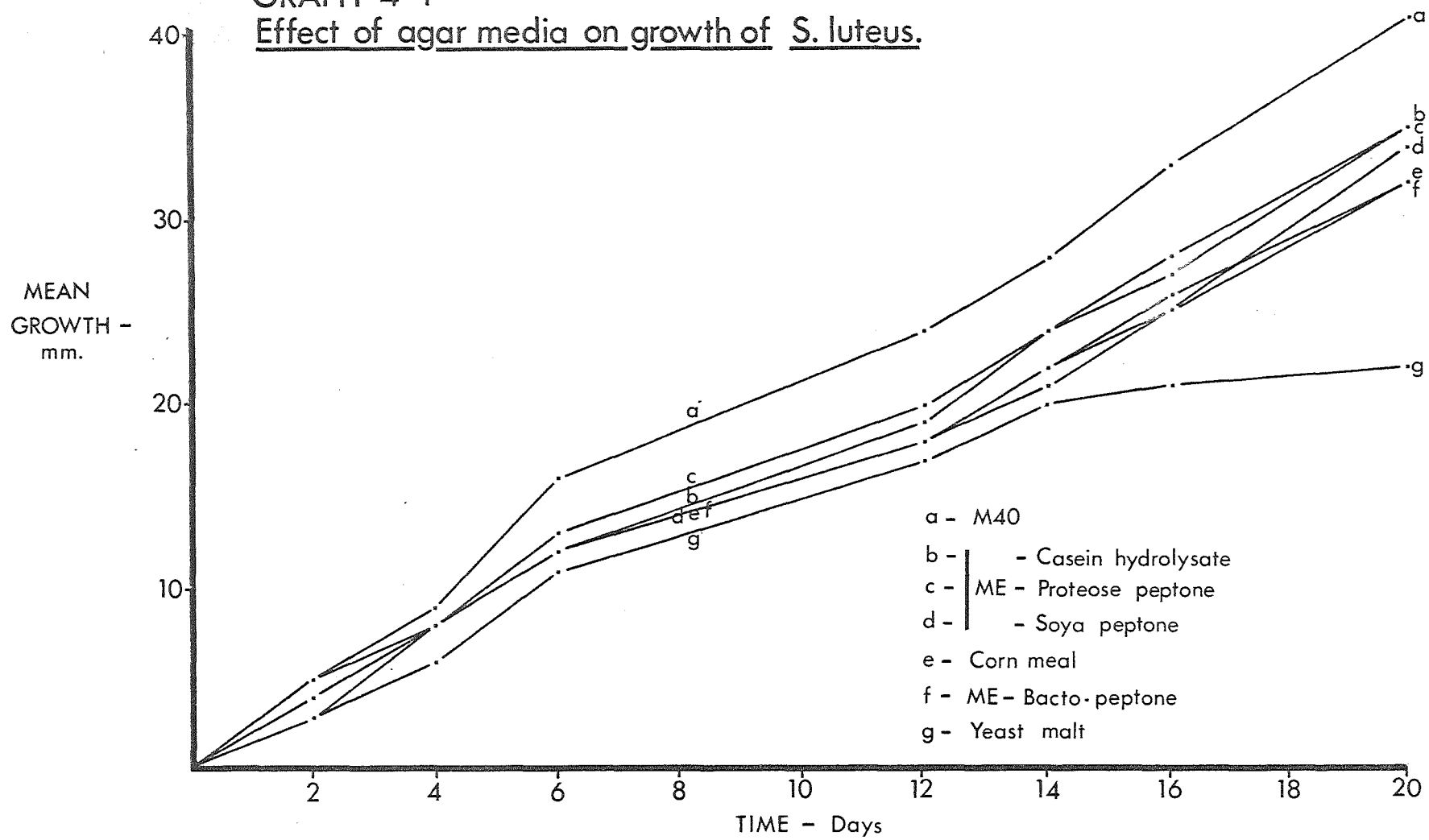
In the agar media sap-hyphae are present (Fig. 4.5) and these may act as nutrient uptake and storage organs.

4.3.2 Effect of Culture Medium

Agar plates of several types used during this experiment were originally made up to test spore germination but, as this was not observed after 3-4 weeks, five spore plates of each medium were inoculated from cultures of isolate Sp.A. (Sporophore isolate A - 13/12/76) and growth increments along four co-ordinates per plate were noted at regular intervals over a period of three weeks. All were incubated in the dark at 25°C. The results are shown in Graph 4.1. On M40 the mycelium grew rapidly producing many aerial hyphae and had the appearance of a thick, woolly mat. The top surface was cinnamon brown/off white and the agar stained a light brown. On Yeast Malt (see Appendix 1) growth was slow, the mycelium was tightly woven and few aerial hyphae were present. The colony surface was white/brown and the agar stained dark brown. Corn meal and M.E. (Proteose Peptone) produced sparse growth with tufts of aerial

GRAPH 4-1

Effect of agar media on growth of S. luteus.



hyphae and little staining. M.E. (Bacto Peptone), M.E. (Casein Hydrolysate) and M.E. (Soya Peptone) had more aerial growth, still tufted, and light brown staining of the medium. Growth was found to be very restricted on Czapek-Dox medium.

In terms of production of hyphal material, M40 was superior to the other media and also gave greater radial growth on the plates. This medium was therefore used for all culturing work during this study and typical colony growth on M40 plates are shown in Fig. 4.6.

4.3.3 Effect of pH

Culturing and spore germination work necessitated determination of the optimum pH for the growth of S. luteus. The fungus was grown on M40 A broth adjusted to pH s 3 — 8 (before autoclaving) with 0.1 N HCl or 0.1 N NaOH. Five replicate flasks were used (20 ml/100 ml flask). Additional flasks were prepared to monitor pH changes after autoclaving and this was also recorded at the termination of the study. Inoculation and weighing is described in Section 4.2.2.

Autoclaving changed the pH of the medium considerably in all treatments except those for pH 4 - 5 (Graph 4.2). Optimum growth of S. luteus occurred at approximately pH 5.0 (Graph 4.3). Modess (1941) calculated the pH optimum for S. luteus to be 5.5 and those of S. granulatus and Amanita muscaria to be 5.1 and 4.6 respectively but the medium used was considerably different to M40 A. The pH of M40 A after autoclaving (5.1) is therefore optimal for fungal growth.

FIGURE 4.6 Pure cultures of S. luteus on M40 agar.

A Single culture derived from sporophore tissue.

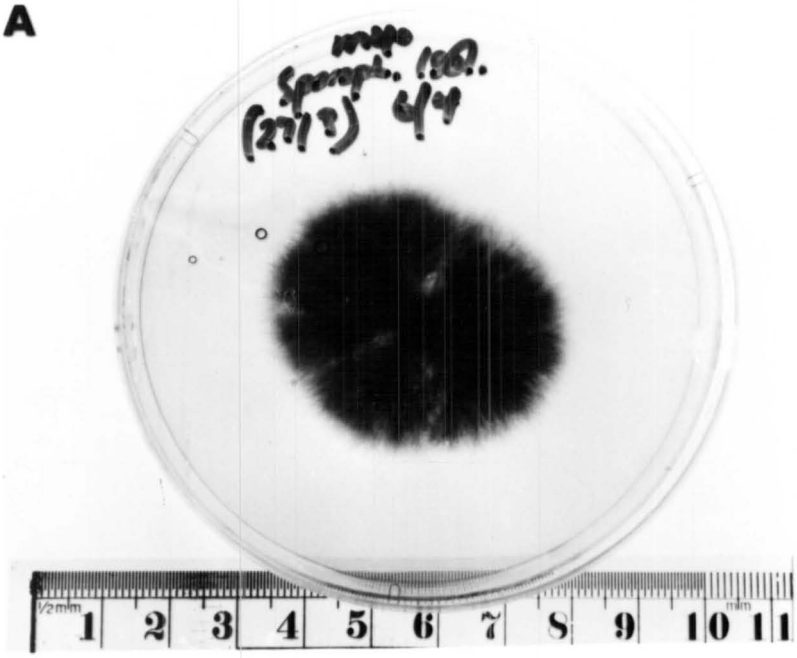
B Cultures consisting of isolates from:

1 Sporophore tissue

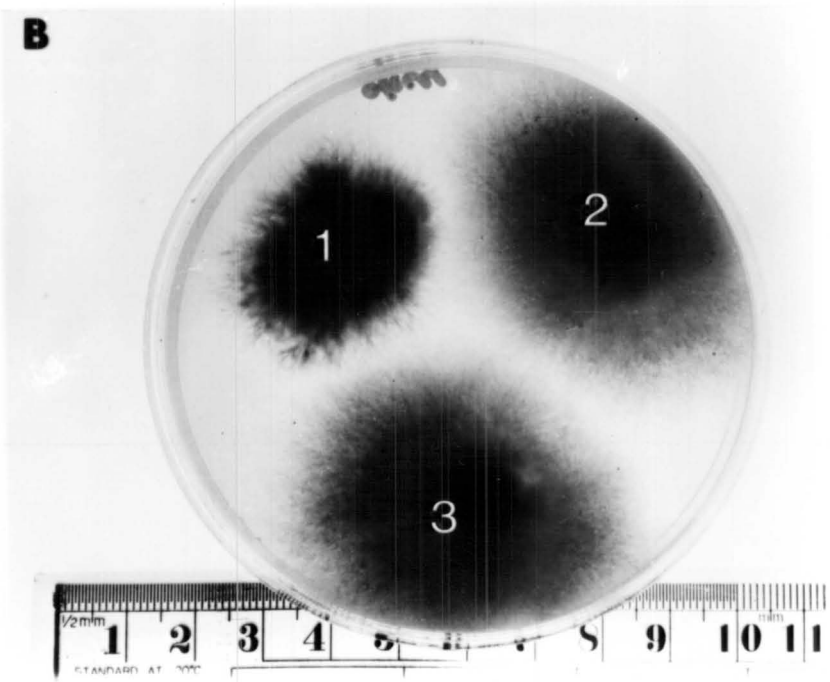
2 Germinated basidiospores

3 Mycorrhizal roots

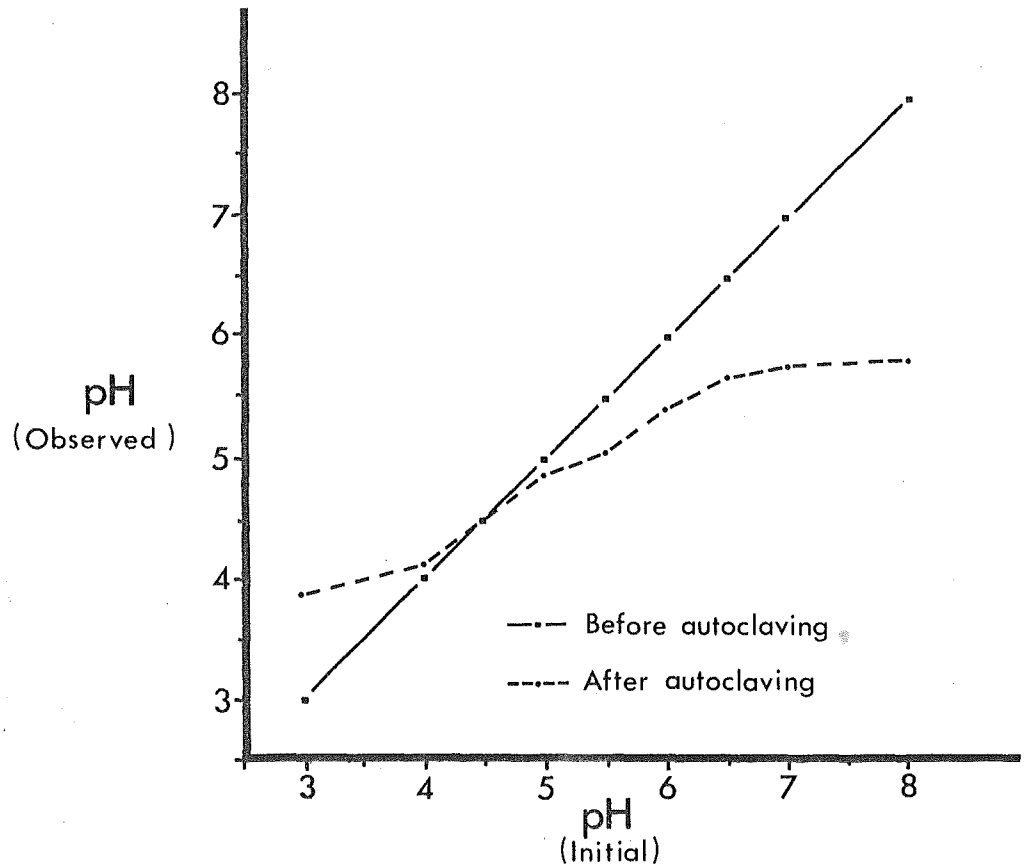
4-6 A



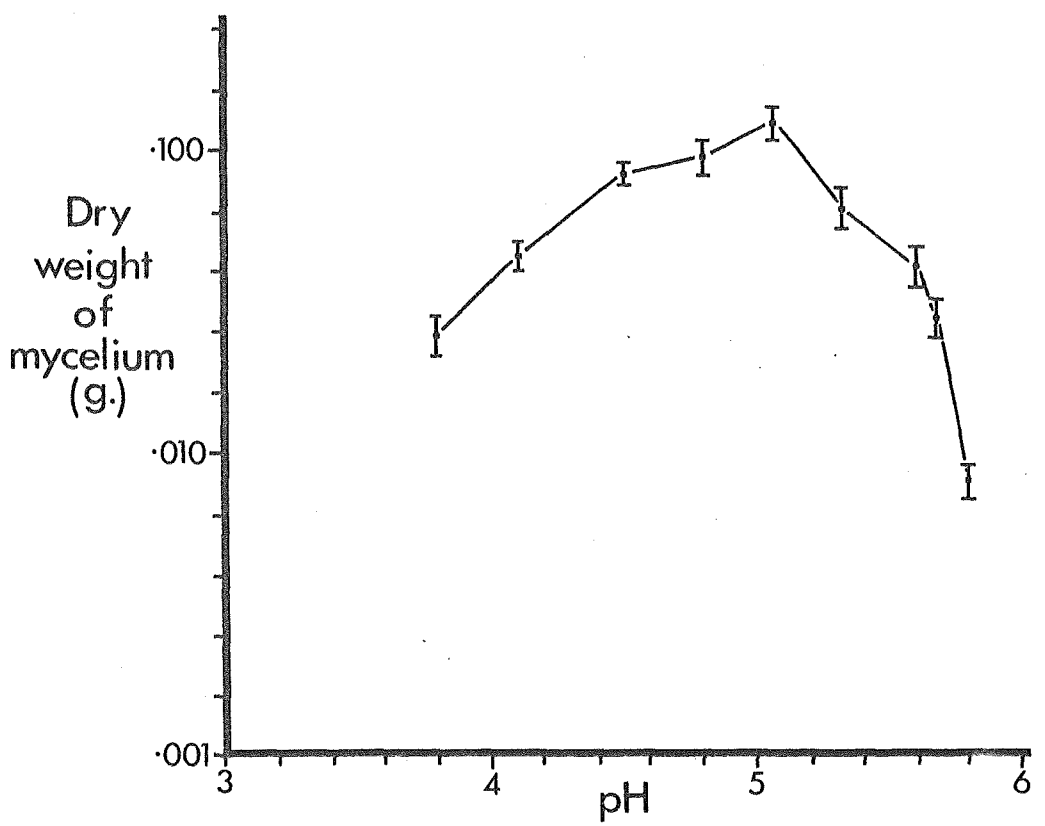
B



GRAPH 4-2
pH change in M40 medium during autoclaving.



GRAPH 4-3
Optimum pH for growth of S. luteus.



The acidophilic nature of S. luteus is evident from Graph 4.3 since growth decreases rapidly as the pH rises. Where growth was rapid near the optimum pH, it was found that the greatest change in the pH of the medium occurred and that this change increased the acidity. A feature of mycelial growth throughout this experiment was the production of the brown exudate which increased in intensity as the medium became more alkaline or where growth was particularly rapid. The absorbance of the exudates was measured by spectrophotometer (Bausch and Lomb Spectronic 20) at 400 nm (Table 4.1).

TABLE 4.1 EFFECT OF pH ON BROWN PIGMENT INTENSITY

pH	3	4	4.5	5	5.5	6	6.5	7	8
Percent Absorbance	0.26	0.66	0.95	1.1	1.3	1.5	1.8	2.0	2.0-∞

The pH 8 sample was also tested over the visible spectrum and showed two peaks at 870 nm and 350 nm.

Samples of soils found at Broken River were also tested for their pH. Twenty grams of soil were added to 50 ml distilled water, shaken 15 minutes then left 30 minutes to settle before readings were taken from each sample. The mean pH was 6.0. This, as shown above, is not conducive to growth of S. luteus and it has been noted that mycorrhizal

fungi do not frequent soils lacking litter or well developed root systems. Where soil litter layers are present, however, more acidic conditions prevail (pH 5.0 - 5.4) and mycelium can readily be found near tree plots in the organic horizon.

4.3.4 Effect of Nitrogen

Inorganic N sources such as ammonium compounds usually stimulate growth of many of the fungi found in association with higher plants. Many mycorrhizal fungi cannot utilise nitrate (Norkrans, 1950) although Cenococcum graniforme is able to in the absence of any other N source (Mikola, 1948). Amino acids are reported to be even better sources of N than ammonium compounds (Melin and Mikola, 1948; Melin and Norkrans, 1948; Mikola, 1948). Glutamine, in particular, is an important organic form of N assimilated by mycorrhizal fungi (Carrodus, 1966, 1967).

The following N sources were investigated for their effect on the growth of S. luteus using medium G (Appendix 1):

1 Control - no N.
2 KNO_3
3 $NaNO_3$
4 NH_4NO_3
5 NH_4Cl
6 $(CHOH\ COONH_4)_2$
7 $NH_2\ CO\ NH_2$
8 Neopeptone

Each test solution contained 0.5 g/l of the appropriate N source, and five replicates were used per treatment (20 ml/100 ml flask). The pH changes observed are shown in Table 4.2.

TABLE 4.2 pH CHANGES DURING N SOURCES STUDY

Treatment	pH - before autoclaving	pH - after autoclaving	pH - termination of experiment
1	4.1	4.3	4.2
2	4.3	4.2	4.6
3	4.3	4.3	4.3
4	4.3	4.3	4.0
5	4.3	4.3	4.0
6	4.1	5.0	4.2
7	5.6	6.5	4.6
8	5.9	5.2	4.5

The nitrate sources did not support good growth (Histogram 4.1) but NH_4Cl and ammonium tartrate gave a better response. The latter medium, with two ammonium Ns, did not stimulate growth as much as that containing the two N compound urea. The best N sources were urea and neopeptone and again the medium became acidic during the fungal growth. Many aerial hyphae were produced in these treatments and the medium also

HISTOGRAM 4-1 Treatments comprised the following nitrogen sources:

1 Control (Ff)

2 KNO_3 (Ee)

3 $NaNO_3$ (Ff)

4 NH_4NO_3 (Ff)

5 NH_4Cl (Dd)

6 $(CHOH \cdot COOHNH_4)_2$ (Cc)

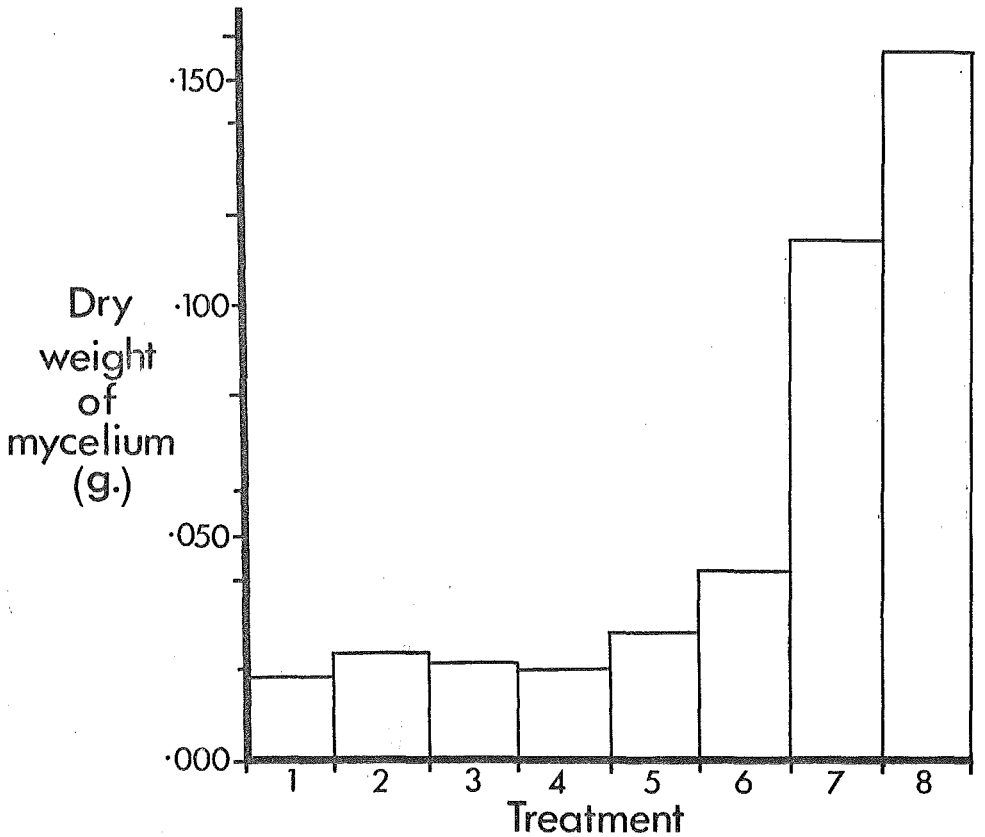
7 $NH_2 \cdot CO \cdot NH_2$ (Bb)

8 Neopeptone (Aa)

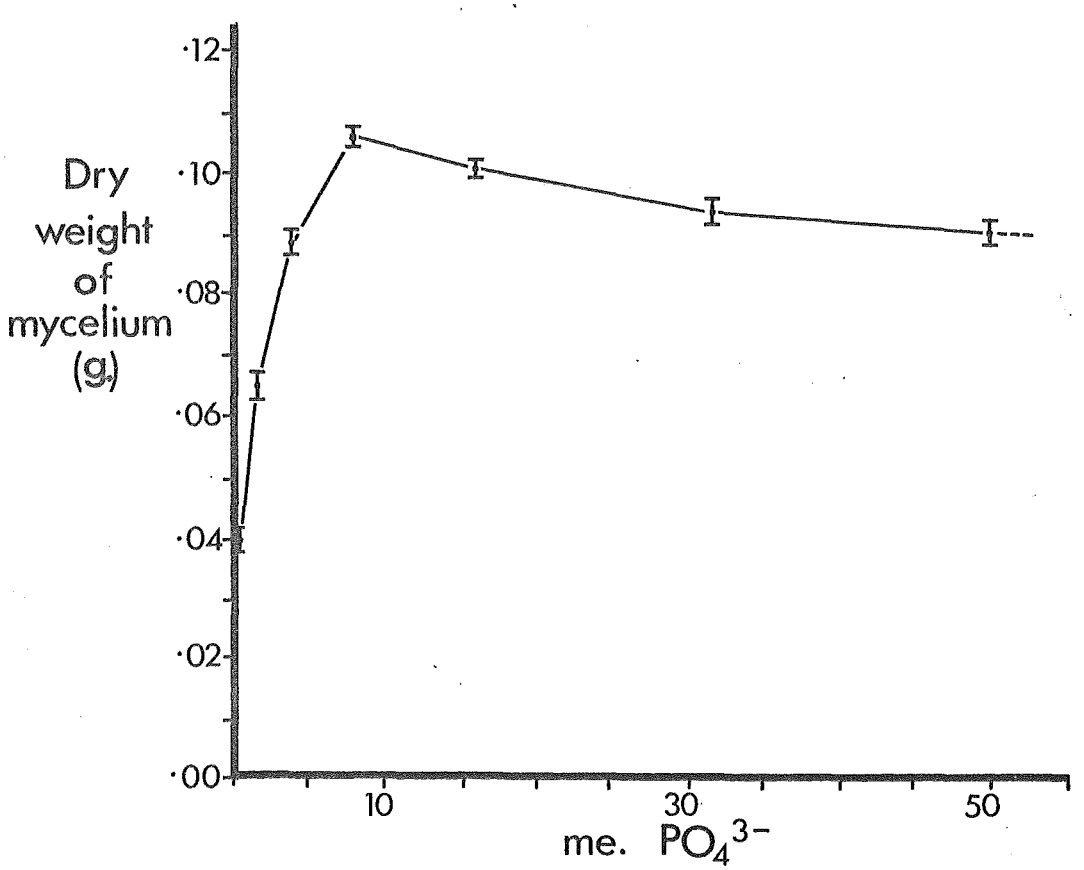
Std error = 6.3×10^{-4}

Significant difference and ranking are given in brackets.

HISTOGRAM 4-1
Effect of nitrogen sources on growth of S. luteus.



GRAPH 4-4
Effect of phosphate level on growth of S. luteus.



stained brown. *In vitro* *S. luteus* rapidly assimilates ammonium N and although it is difficult to relate this to the soil situation it is probable that, in conjunction with ammonifying micro-organisms in the rhizosphere, mycorrhizal fungi can utilise ammonium ions normally absorbed on colloidal particles in the upper soil horizons. The work of Richards and Voight (1964), Richards (1973) and Lewis and Harley (1965) indicated that N-fixing bacteria can be encouraged in the mycorrhizal rhizosphere and that this may be due to production of mannitol, an important carbon source for many N-fixers, by ectotrophic mycorrhizas.

Some of these N sources were also studied for their effects on spore germination as discussed in Chapter V.

4.3.5 Effect of Phosphorus

The ability of trees to assimilate P is a particularly important part of their nutrition and the marked effects of mycorrhizas on P uptake and tree growth were first shown by Hatch in 1937. It has been shown that *S. luteus* stimulates uptake of P by various host trees, even when present in a poorly soluble form. In *P. sylvestris* (Ritter and Lyr, 1963; Mejstrik, 1975) and *P. radiata* (Bowen and Theodorou, 1967; Mejstrik and Krause, 1973) the symbiont significantly increased uptake of P especially from organic forms (estimated to be between 80-90 percent of the phosphate in *P. radiata* litter.) Also, when using soluble P(KH_2PO_4), iron phosphate, rock phosphate, apatite and humus, *S. luteus* showed increased mobilisation of all forms to provide an

available source of P for the tree. Fowells and Krause (1959) showed the optimum levels of P for P. taeda were in the range 1-25 ppm (40-600 ppm according to an analysis by Woodwell, 1959) and it would seem that low levels of P are conducive to mycorrhiza-like root morphology. The optimum P level for growth of S. luteus was investigated using soluble KH_2PO_4 in the following quantities (Table 4.3).

TABLE 4.3 pH CHANGES DURING P STUDY

Milli-equivalents PO_4^{3-}/ treatment	pH - before autoclaving	pH - after autoclaving	pH - termination of experiment
0	6.0	5.8	3.4
0.5	5.7	5.6	3.4
1.0	5.7	5.5	3.5
1.5	5.5	5.4	3.6
2.0	5.5	5.4	3.6
4.0	5.6	5.4	3.6
8.0	5.4	5.3	3.9
16.0	5.3	5.3	3.9
32.0	5.1	5.2	4.1
96.0	4.8	4.8	4.2

The results are shown in Graph 4.4 where it can be seen that

maximum growth occurs at approximately 8 me P (0.005 percent PO_4^{3-}). Normal total P levels in a good arable soil may reach 0.2 percent although only 0.01 - 0.015 percent of this is available soluble P (Davies, 1939). Comparatively, the level of P supporting best growth of S. luteus is very low. The levels of P found in soils from the Broken River area are shown below (Table 4.4) and it is evident that the amount of total P is low, even in the upper horizons.

TABLE 4.4 PHOSPHATE LEVELS IN A HIGH COUNTRY YELLOW BROWN EARTH

Horizon: Depth:	A_{11} 0-6 cm	A_{12} 6-23 cm	A_B 23-30 cm	(B) 30-40 cm	C 40-75 cm
Total P - Mg percent	131	123	97	99	74
Organic P - Mg percent	77	70	48	36	13
Inorganic P - Mg percent	54	53	49	63	61
Total Extracted P - Mg percent	61	58	49.5	61.5	56.7
Percentage P retention	64	70	74	91	74

Puketeraki Silt Loam (Strongly leached H.C.Y.B.E.)
NZ DSIR Soil Bureau Bulletin 26, p 45 - (1968).

This table shows that, because of the high retention of P (20-30 percent of the soil is composed of clay), the low levels of available P will have a repressive effect on tree growth. Although it is difficult to extrapolate from *in vitro* studies to the soil situation, low levels of P would appear to be beneficial to growth of the mycorrhizal fungus and it is known that insoluble P can be utilised (Bowen and Theodorou, 1967). In soils deficient in P the presence of mycorrhizas would insure an adequate supply since plant response to P levels only occurs above 0.025 percent (Davies, 1939). Mycorrhizas having good ability to mobilise insoluble P found in young soils should therefore be selected for inoculation programmes. Phosphate fate, once assimilated by the fungus, is discussed in Chapter IX.

4.3.6 Effect of Organic Acids

Actively growing roots produce their own characteristic combinations of exudates and organic acids are major constituents of these. Since the 1940s it has become increasingly apparent that these exudates are of great importance to the microflora in the rhizosphere, providing energy sources and essential micro-requirements (particularly the B vitamin group for which many endo- and ectomycorrhizal fungi are partial heterotrophs). The effects of several organic acids on the growth of *S. luteus* were investigated. These were millipore filtered and then added to a base solution at a concentration of 5.0 g/l, except for Malic acid (2.5 g/l) and Shikimic acid (1.29 g/l), as the sole carbon source.

The following organic acids were used:

1 Control - no organic acid
2 Acetic acid
3 Citric acid
4 Fumaric acid
5 Malic acid
6 Malonic acid
7 Oxalic acid
8 Shikimic acid
9 Succinic acid

The results of this study are shown in Histogram 4.2. Good growth occurred in the presence of both malic and succinic acids and to a lesser extent with fumaric acid. These are all four-carbon compounds of the Krebs cycle which are interconvertible and play an important role in the formation of amino acids such as aspartate and ornithine cycle amides. Some growth occurred with citric, oxalic and malonic acids but was poor on the others. Melin (1954) showed that low levels of glutamic and aspartic acids were optimum for growth of S. luteus. The ability of this mycorrhizal fungus to utilise organic acids, even in small quantities, indicates that its growth may be stimulated on the host root rhizoplane by the tree exudates. These acids may also be important components of Melin's "M-factor" and thus have roles in both basidiospore germination and fungal growth.

HISTOGRAM 4-2 Treatments comprised the following organic acids:

1 Control (Ff)
2 Acetic acid (Ff)
3 Citric acid (Dd)
4 Fumaric acid (Cc)
5 Malic acid (Bb)
6 Malonic acid (Ee)
7 Oxalic acid (Ee)
8 Shikimic acid (Ff)
9 Succinic acid (Aa)

Std Error = $\pm$ 6.6 x 10^{-4}

Significant difference and ranking are given in brackets.

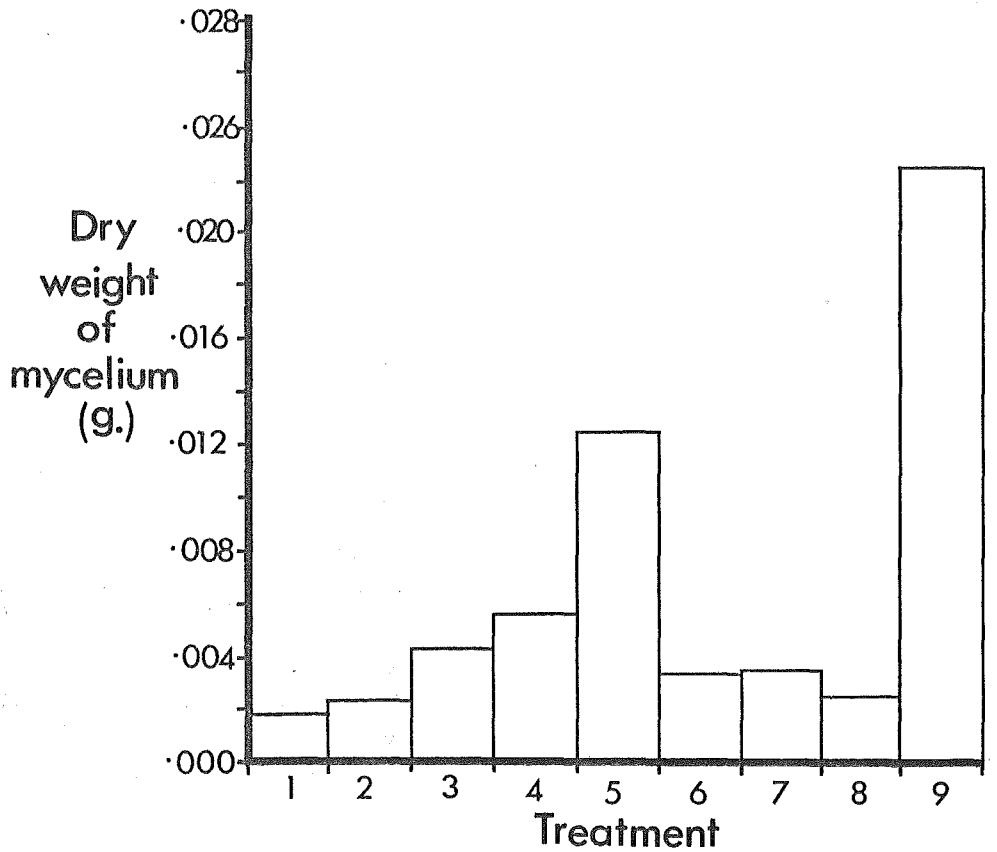
HISTOGRAM 4-3 Treatments comprised the following carbohydrates:

1 Control (Hh)
2 Glucose (Ccb)
3 Fructose (Ff)
4 Mannose (Bb)
5 Ribose (Dd)
6 Sucrose (Ee)
7 Maltose (Aa)
8 Mannitol (Cc)
9 Lactose (Ff)
10 Methyl-cellulose (Gg)
11 Malt extract (Aa)

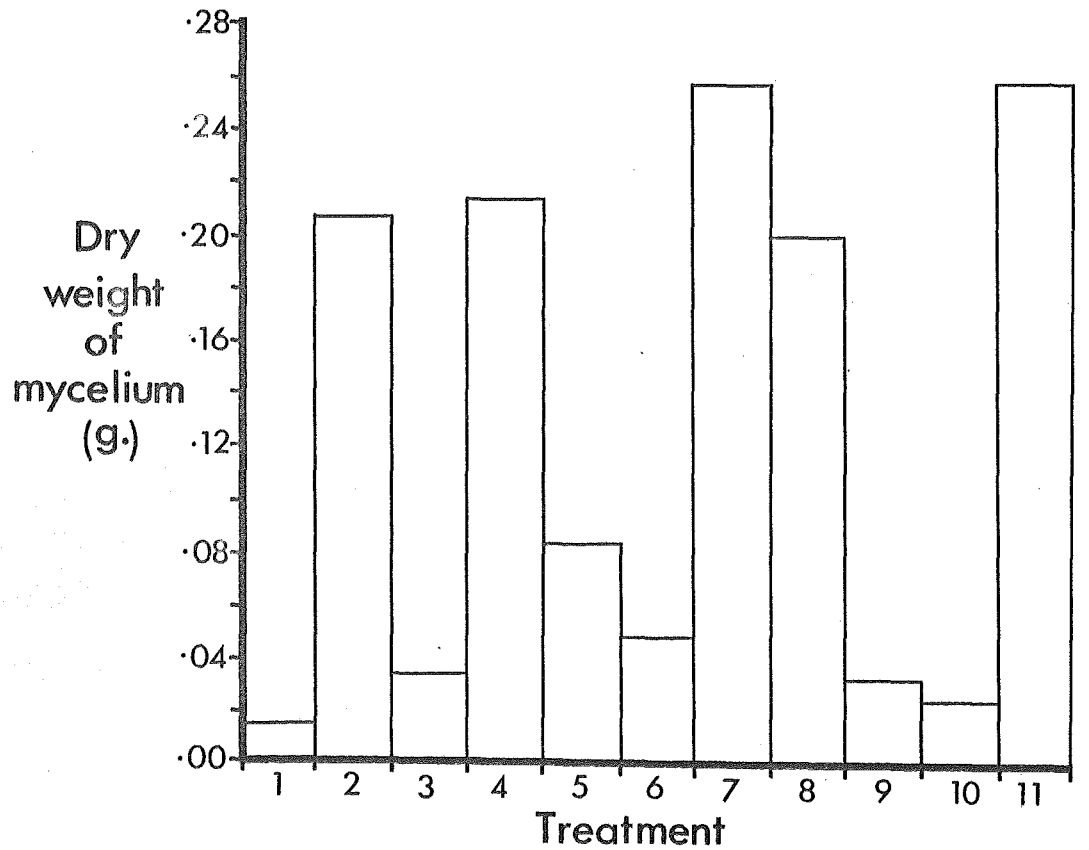
Std Error = $\pm$ 8.5 x 10^{-4}

Significant difference and ranking are given in brackets.

HISTOGRAM 4-2
Effect of organic acids on growth of S. luteus.



HISTOGRAM 4-3
Effect of carbohydrates on growth of S. luteus.



4.3.7 Effect of Carbohydrates

Carbohydrate nutrition of Boletus species has been studied by many workers, most of whom observed better growth on simple carbon compounds. Ferry and Das (1968) indicated that glucose was the best carbon source and that mannose is often as good but fructose, galactose and xylose were all poor. Some utilization of dissacharides was evident, particularly of maltose, sucrose or malate. Utilization of polysaccharides was generally poor. B. luteus was shown to utilize such diverse carbon sources as glucose, mannitol, cellobiose, mannose, maltose, sucrose and fructose (in order of decreasing growth response). Five isolates of S. luteus were studied for their growth on ten carbon sources. Three of the isolates used were derived from sporophore tissue (Sp. A, Sp. B - isolated 13/12/76 and Br. Rv. A - isolated 1/3/75) and two were derived from mycorrhizal roots (R 1 - isolated 2/8/76 and R 2 - isolated 29/11/76). When studied, the most recent of these isolates were approximately six months old. Medium E (Appendix 1) was used with 5 g/l of carbon source being added and having five replicate flasks per isolate per treatment. The following carbon sources were used (Table 4.5).

TABLE 4.5 pH CHANGES DURING CARBON SOURCES STUDY

	Carbon Source	Fungal Isolate Br.Rv. A	Sp A	Sp B	R 1	R 2
1	Control	6.8	6.8	6.8	6.8	6.8
2	Glucose	3.3	3.4	3.4	3.3	3.3
3	Fructose	6.7	6.7	6.6	6.7	6.6
4	Mannose	3.2	3.2	3.3	3.3	3.3
5	Ribose	6.8	6.8	6.7	6.7	6.8
6	Sucrose	7.3	7.3	7.3	7.3	7.3
7	Maltose	3.4	3.3	3.4	3.4	3.4
8	Mannitol	3.5	3.5	3.5	3.5	3.5
9	Lactose	6.6	6.8	6.8	6.6	6.7
10	Methyl-cellulose	6.6	6.7	6.6	6.6	6.7
11	Malt Extract	3.9	3.9	3.9	3.9	4.1

The results are shown in Histogram 4.3. The best carbon sources were maltose, malt extract, mannose, mannitol and glucose, with ribose and sucrose giving poor growth. Maltose is a component of tree root exudates (Bowen and Theodorou, 1973) as is glucose. Mannose and mannitol (with six carbon chains) may also be important in plant exudates. Norkrans (1950), Palmer and Hacskaylo (1970) and Melin (1925) have all shown that both mannose and mannitol can be assimilated by various mycorrhizal fungi, including

B. variegatus, B. elegans, S. cothurnatus, S. punctipes, Tricholoma species, C. graniforme, R. roseolus and A. rubescens. The five isolates of S. luteus gave uniform growth with the different treatments used and are therefore similar in their requirement for carbon. Glucose did not support good growth, contrary to that indicated by Ferry and Das (1968), however it is possible that glucose growth repression may have occurred.

4.3.8 Discussion

Isolation of S. luteus in pure culture can be readily achieved using the technique outlined. To maintain these cultures the fungus should be grown on a malt extract based medium. Correct pH balance is also important when growing S. luteus and M40 A agar/broth, with a pH (autoclaved) of 5.1, satisfies both these requirements. The acidophilic nature of S. luteus was evident, a feature shown by most mycorrhizal fungi, and this generally corresponds well with the pH of forest soils (Modess, 1941; Melin, 1953). The pH has been shown not only to affect host tree growth and mycorrhizal infection in the forest system, but also the types of mycorrhizas found on the roots (Theodorou and Bowen, 1969). Optimum temperature for in vitro growth of most mycorrhizal fungi is approximately 20-25°C (Theodorou and Bowen, 1971a; Slankis, 1974) and all culturing work during this study was successful at 25°C.

It is evident that nitrate cannot support growth of S. luteus, however, Theodorou and Bowen (1969) have shown

that other mycorrhizal fungi, such as R. luteolus, S. granulatus and C. graniforme, can grow on this substrate (cf Richards, 1961, 1965). Both urea and neopeptone were good sources of nitrogen and in the soil it is probable that there is a close relationship between the ectomycorrhiza and nitrogen-fixing bacteria in the rhizosphere, this directly benefitting the host tree (Richards, 1973, 1974). S. luteus can survive on very low levels of phosphorous and the presence of the mycorrhizas on trees in P deficient soils (as at Broken River) is advantageous. Insoluble forms of P are mobilised by the mycorrhiza and stored, or transferred to the tree as required (Bowen and Theodorou, 1967; Ashford et al. 1975).

When considering interactions between host and fungus in mycorrhizal symbiosis, it can be seen that S. luteus will benefit from organic acids known to be exudates of the phanerogam partner. Those involved are associated with the metabolically essential, energy releasing, Krebs cycle and are effective even in small quantities.

A wide range of mono- and disaccharide carbohydrates can be utilized by S. luteus. This was also shown by Lamb (1974) who found that S. luteus could adapt to the largest number of carbon sources and utilized the greatest number in the absence of added glucose 'starter'. Conversely, S. grevillei was found to have the most specific carbon source requirements of the fungi tested. The diverse carbohydrates available to S. luteus could, therefore, provide the fungus with adaptability over a wide range of hosts and conditions when considering its assimilation of

photosynthetic products.

If S. luteus could be induced to produce sporophores in pure culture, many of the problems encountered with inoculum preparation would be solved. In particular, readily available inoculum obtained prior to seed sowing would ensure that spores were viable. Further investigation of the nutritional requirements of the fungus may indicate the factors that are necessary to initiate fruiting. A medium supplemented with compounds such as maltose, neopeptone or urea, malic or succinic acids and vitamins such as thiamine could be used. With readily available sporophores in pure culture the necessity for spore storage, with its effects on germination (see Chapter VI), would be overcome.

CHAPTER V

BASIDIOSPORE GERMINATION

5.1 INTRODUCTION

Basidiospores appear to be the major disseminating agent for most mycorrhizal fungi. The considerable difficulty in obtaining mycorrhizas on developing seedlings where a soil has no apparent fungal symbiont would indicate that, although vast numbers of spores are produced, their viability is questionable or their effective dissemination by wind is limited (Lamb, 1974). Trees may survive a number of years before becoming mycorrhizal even where sporophores are commonly found nearby. Spores already in the soil may therefore be dormant (or non-viable) and infection could occur by their germination or by root contact with mycelium or freshly released spores. Various factors are known to influence spore germination, including a requirement for the vitamins thiamine and biotin (Harley and Lewis, 1969; Rovira and Harris, 1961; Robbins, 1952) and the effects of cold temperature (Sussman and Halvorson, 1966), ultra-violet (UV) light (Trione and Leach, 1969; Tinline *et al*. 1960), the presence of stimulatory organisms such as the yeast *Rhodotorula* (Melin, 1953) or living mycelium of the fungi concerned (Losel, 1964). In addition, many chemical compounds have been implicated as germination stimulants, some of which are known to be components of root exudates

(Sussman and Halvorson, 1966; Bowen and Theodorou, 1973). If a germination stimulant could be found, suitable inoculation procedures incorporating this might be devised to assist direct infection of the host. Problems still exist with the germination of basidiomycete spores, *in vitro* ($\leqslant$ 0.01 percent in many cases) further hindering viability studies. During this study spore germination and the effects of some possible germination stimulants were investigated.

5.2 MATERIALS AND METHODS

Spores were collected by spore drops directly onto agar surfaces in petri dishes. To maintain sterile conditions small sections of the hymenial tissue from sporophores were removed and, in a sterile cabinet, attached to the lids of petri dishes by a vaseline smear. After suitable spore drop periods (usually 2-4 hours) the lids were removed and replaced with new sterile ones. These experiments could only be conducted during the collecting season because of the dependence on fresh sporophores, thus it was difficult to obtain uniformity between results with spores from the three seasons studied. There was also a limit to the amount of hymenial tissue obtained from each sporophore for testing. The variation in spore dormancy necessitated using spores from several sporophores and the germination characteristics of these were observed during this study. All treatments were incubated at 25^{o}C in the dark and spore germination was assessed microscopically at intervals. Spores with germ tubes greater in length than the diameter of the swollen

spore were considered to be germinated. Mycelial extracts and spore washings were initially investigated as possible germination stimulants. The mycelial extracts were obtained from S. luteus cultures grown on M40 broth. A 20 ml aliquot of distilled water was used to wash 0.5 g of spores on a millipore filter several times and both the extract and spore wash solution were then sterilised by millipore filtering.

A study of the effects of UV light on spore germination was also made using semi-aseptic synthesis techniques. Seeds of P. mugo (FRES 74/177) were obtained from the Forest Service (FRI - Rangiora), surface sterilised in 10 vol. H_2O_2 for 1.5-2 hours (modified from Trappe, 1961) and washed in sterile distilled water. This treatment is reported to stimulate seed germination (see Chapter VII). Seeds were planted in clean 15 cm diameter pots containing autoclaved vermiculite and soaked with a sterile solution of nutrient medium H (Appendix 1). Spores collected from fruiting bodies (April 1975) were used as a source of inoculum. They were exposed to either 254 mμ or 365 mμ wavelengths of UV light for two or five minute periods and diluted to the required concentration in distilled water. Two methods of application and two spore concentrations were used:

A 2.0×10^9 spores pipetted in the vicinity of the seed.

B Slurry applied directly to the seed.

Slurry coated seeds had spore counts ranging from 4.0×10^7 to 6.0×10^7/seed, as determined by haemocytometer counts. Six replicate pots were used per treatment with five seeds per pot. Seedlings were thinned and checked for mycorrhizas at one, two and four months. The experiment was conducted in a growth room at 20°C with a 16 hour day. All watering was done using distilled water, no further applications of the nutrient solution being made.

5.3 RESULTS AND DISCUSSION

5.3.1 Basidiospore Characteristics

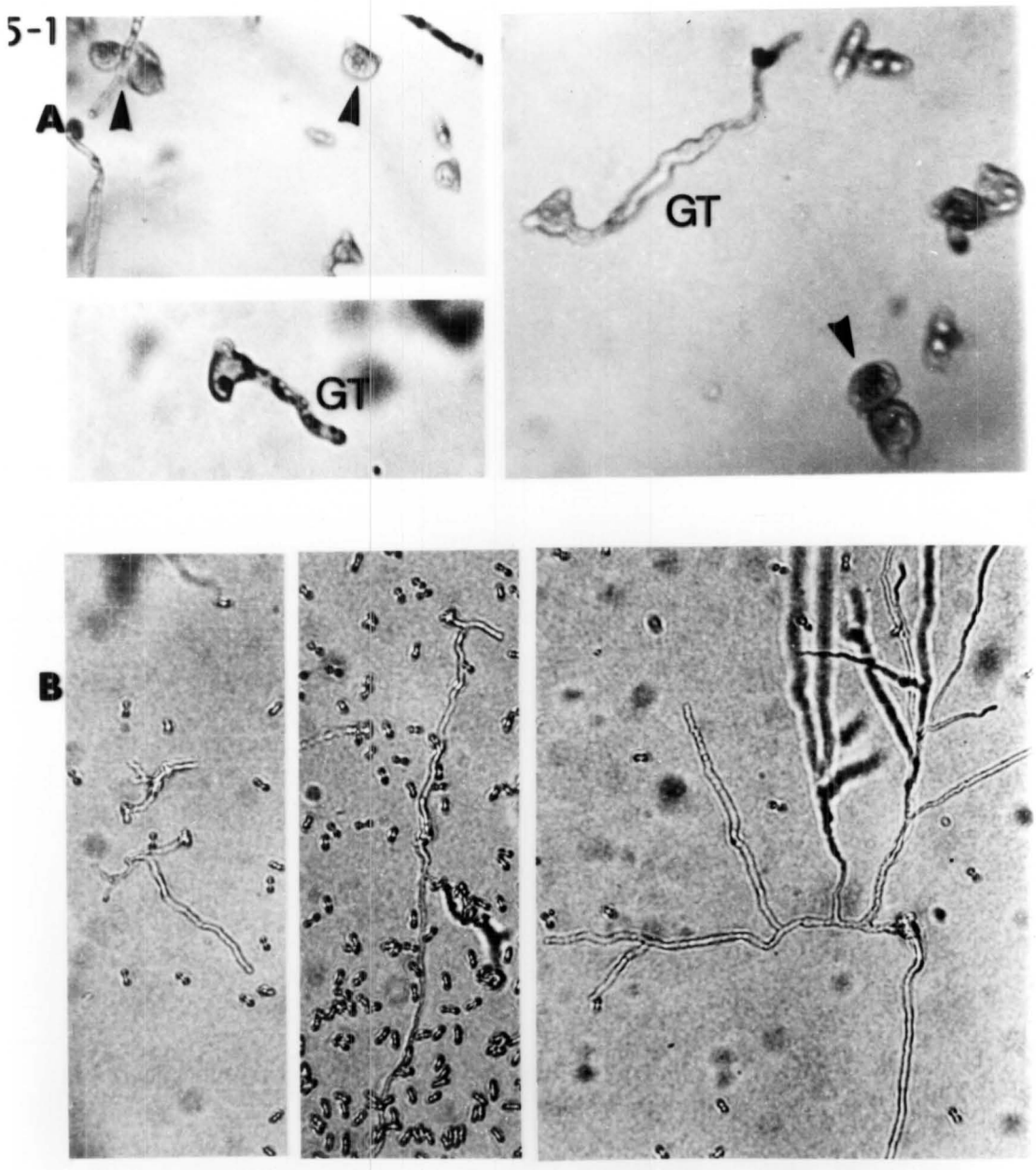
The spores of S. luteus range in size from 7.5-10 µm in length x 2.5-4 µm in width. On initiation of germination they swell, often becoming nearly spherical in shape, their dimensions being approximately 10 x 12.5 µm (Fig. 5.1). The germ-tube generally emerges from the lateral walls of the spore case and not in any specific orientation. A spore 'plug' is found at the point of attachment to the sterigmata and in many other species this serves as an exit point for the germ tube, but not in S. luteus. Internal morphological changes also occur, the most obvious being an increase in size and optical density of bodies seen in the spore lumen (Fig. 5.2). At the time of spore swelling these break down into numerous small, spherical globules just prior to germ-tube emergence (Fig. 5.3). The germ-tubes grow rapidly, exhibiting irregular branching and lacking clamp connections.

FIGURE 5.1 Germination of *S. luteus* basidiospores (light micrographs).

A Swelling of spores prior to germ-tube emergence (arrows) and growth of the germ-tube (GT) from the lateral walls of the spore. x600

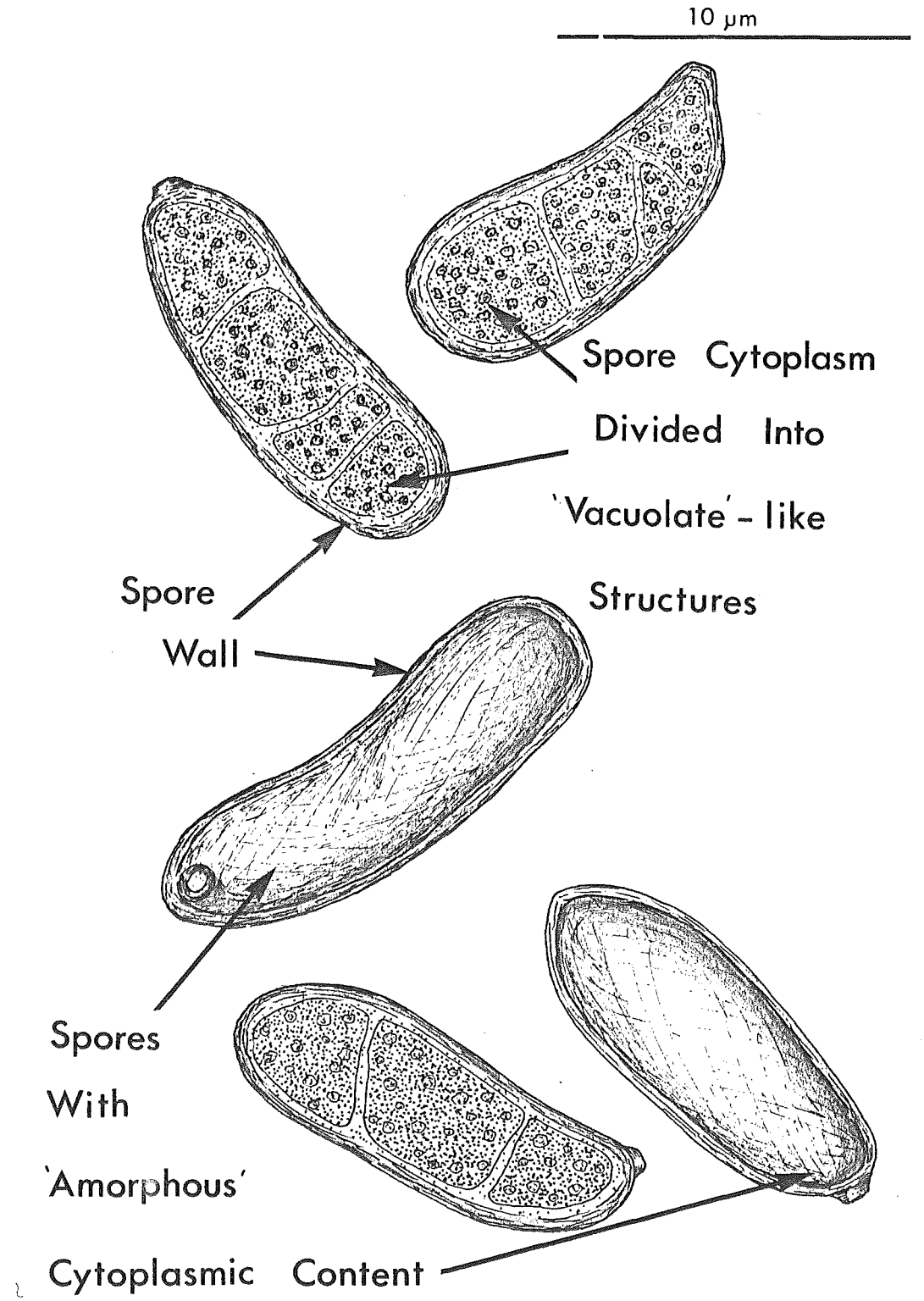
B Later stages in germ-tube growth showing rapid hyphal extension and branching. x200

C Mycelial development of fungus (F) following spore germination. x100


5-1
A
GT
GT
B
C
F
F

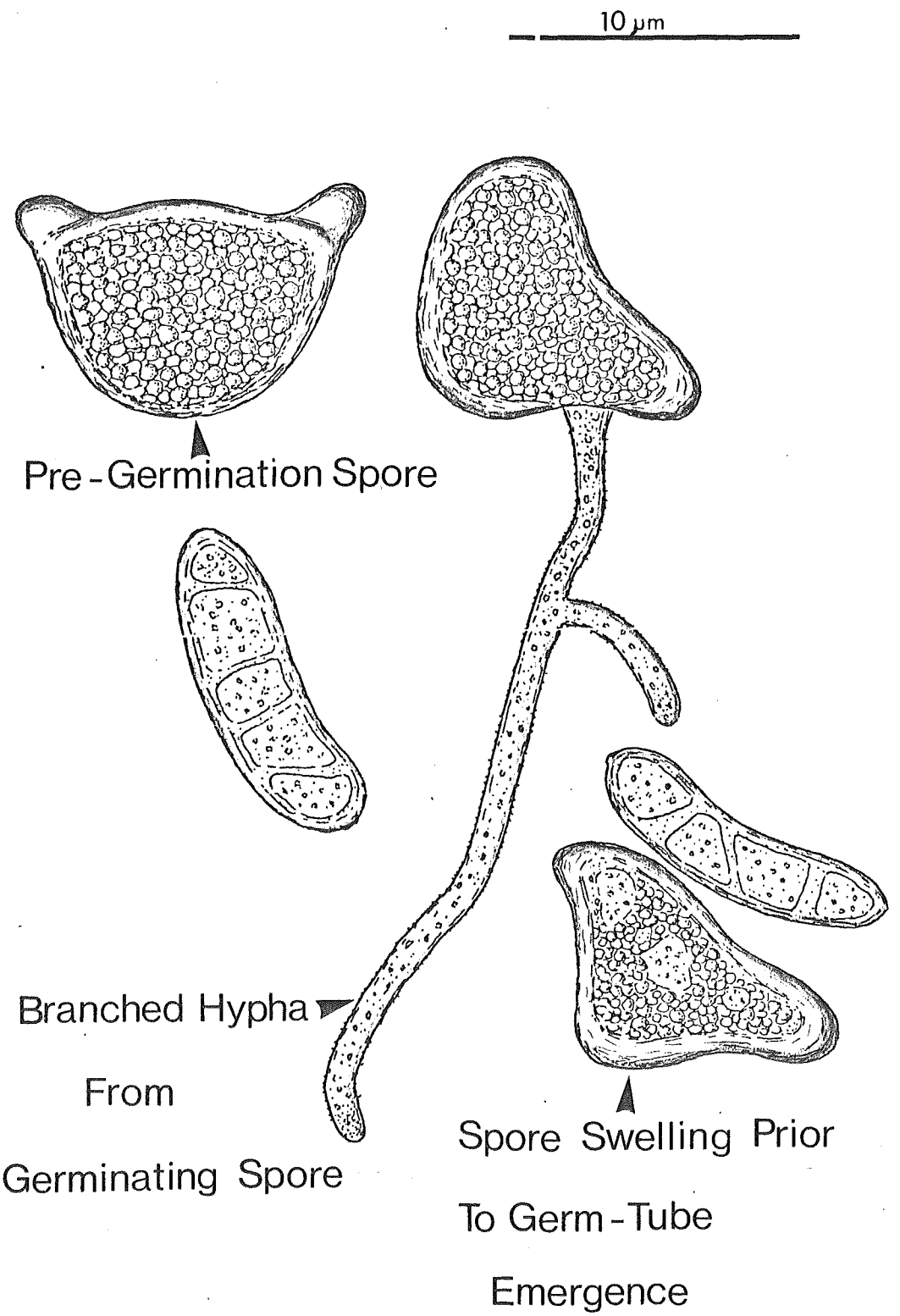
5-2

TYPICAL SPORES - COOL STORED.



5-3

GERMINATING BASIDIOSPORES.



5.3.2 Spore Germination Stimulation

The first factors studied were mycelial extracts and spore washings (see Section 5.2). These were incorporated into M40 agar (as a 10 percent solution). Hymenial tissue from three different sporophores was suspended over the agar to allow spores to fall onto its surface. Germination usually occurred after 2-3 weeks incubation at 25°C, producing the results shown in Fig. 5.4. A wide variation in spore germination percentages between different sporophores is evident. Some spore drops show little germination but others have dense mycelial growth. Spore density effects were also noted. In the control plate, spore drop Y has numerous centres of mycelial growth (each representing single spore germinations) over its whole area and the spore density is low ($\leqslant 10^3$ spores/mm^2). In the two test plates, however, it can be seen that most germination was around the periphery of the spore drops, Z, these having greater spore accumulations. Low spore densities, therefore, appear to favour germination. In total, ten plates were prepared for each of the factors tested and representative plates are shown in Fig. 5.4. The mycelial extract and the spore wash solution increased germination as shown by the numerous centres of mycelial growth. Also the presence of mycelium from germinated spores tended to influence nearby spores, stimulating their germination. This occurred only in low spore density areas and effected spores in the immediate vicinity of those germinating. Later observations on spores washed, stored, or applied to seedlings in distilled water showed that they

FIGURE 5.4 *S. luteus* spore germination; the effect of:

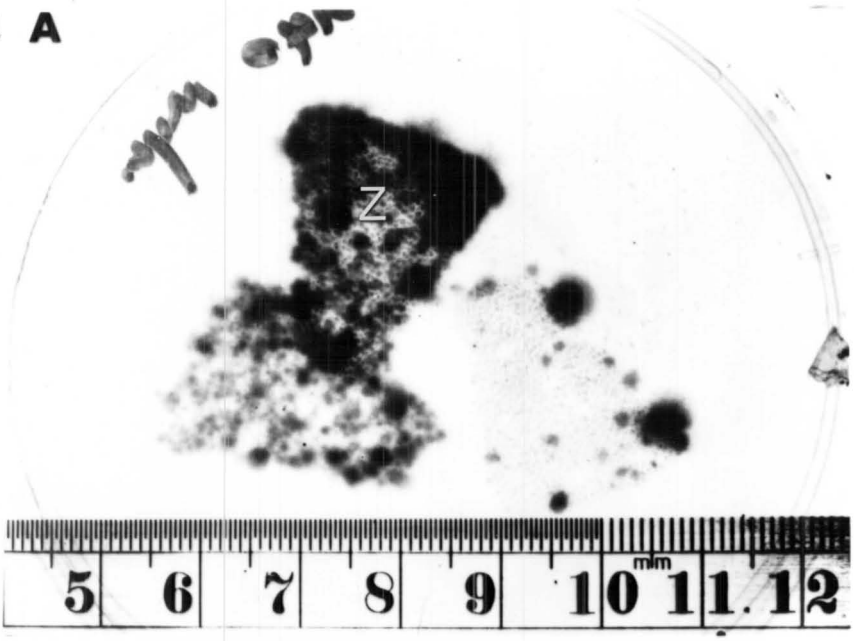
A Mycelial extract incorporated into M40 agar.

B Spore washings incorporated into M40 agar.

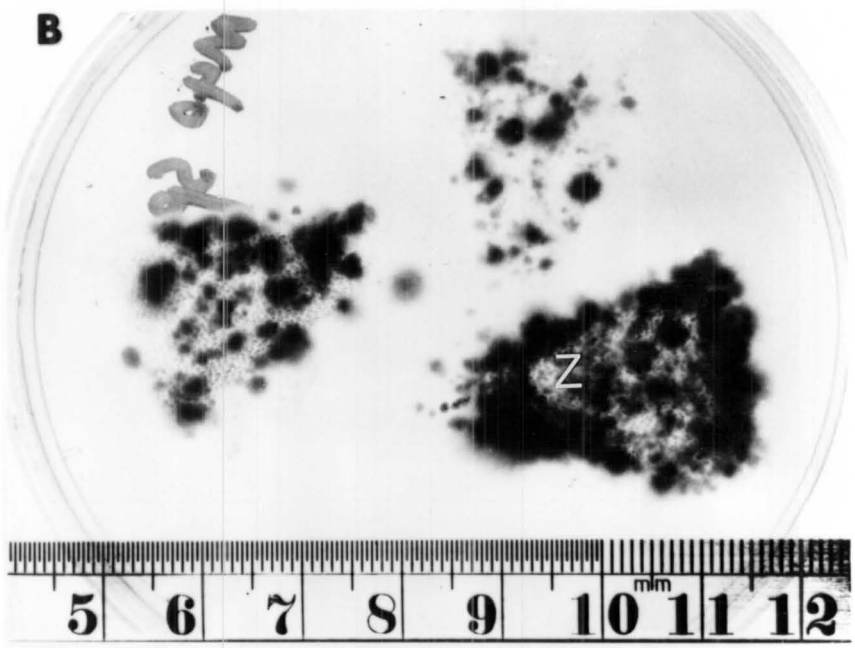
C Control plate showing few germination centres. Note spore drop (Y) with low density of spores shows germination centres over its whole area. Dense spore drops (Z) in plates A and B only show peripheral germination.

Note non-uniformity in germination between spores from different sporophores.

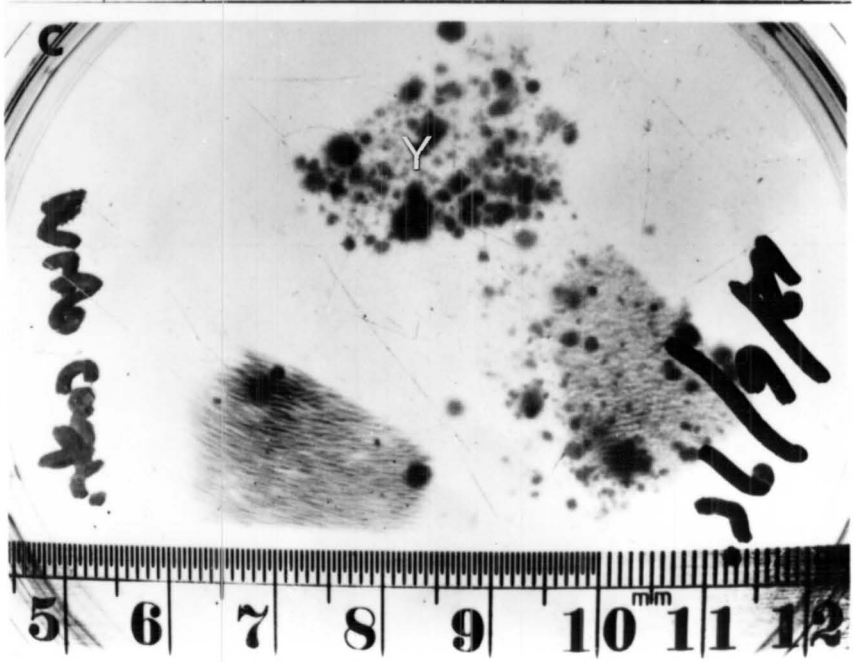
5-4 A



B



C



did not germinate as well as those left dry. Removal of germination stimulating compounds, possibly associated with the spore coat, in the water may account for this.

Nitrogen sources tested in the pure culture experiments were also studied for their effects on spore germination. The N sources NH_4Cl, KNO_3, urea and ammonium tartrate were incorporated into M40 agar in the same proportions as in the pure culture study. Spores were deposited on the agar surface and the plates were incubated at 25°C for 2-3 weeks. Different sporophores were again non-uniform in their spore germination characteristics. Spore germination was stimulated on all test plates in the presence of urea or ammonium tartrate, as compared to NH_4Cl (Fig. 5.5). These N sources, which stimulate pure culture mycelial growth of S. luteus, also have an effect on spore germination. Fewer centres of mycelial growth were seen in the N source study (cf that of mycelial extracts), probably being due to rapid mycelial growth from those spores germinating first obscuring those germinating later.

The effects of the yeast Rhodotorula glutinane (ex Victoria University - 201) were also studied. Cultures of this yeast were grown adjacent to spore drops on M40 agar plates. No evidence for spore germination was seen except on the control plates (Fig. 5.6). It would seem, therefore, that this yeast may be inhibitory to germination.

FIGURE 5.5 The effect of nitrogen sources on *S. luteus* spore germination.

A KNO_3 - very little germination.

B NH_4Cl - normal constituent of M40 = control.

C Urea - good germination stimulation.

D Ammonium tartrate - again many spores stimulated.

Note that spores from different sporophores are again non-uniform in their germination characteristics.

5-5

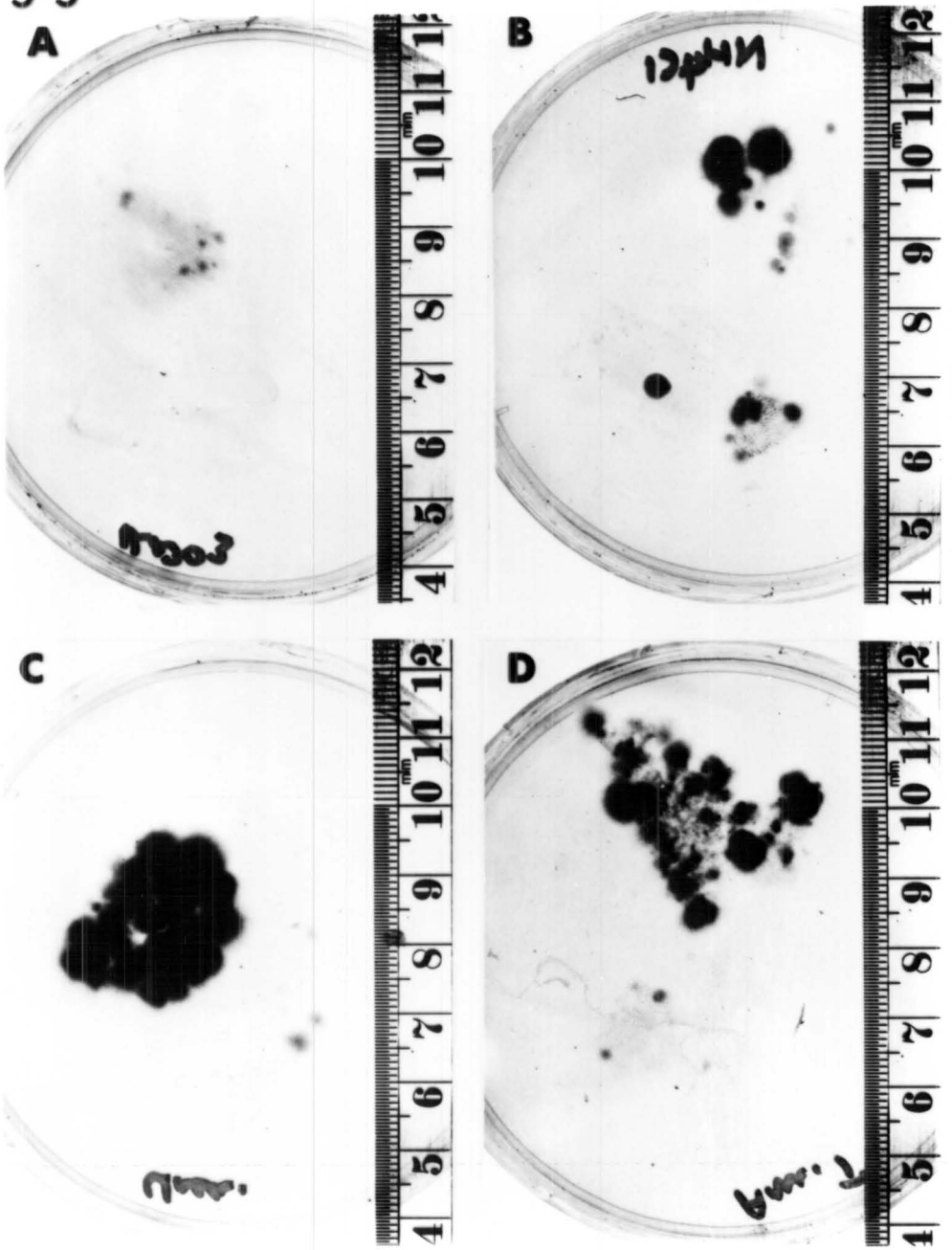
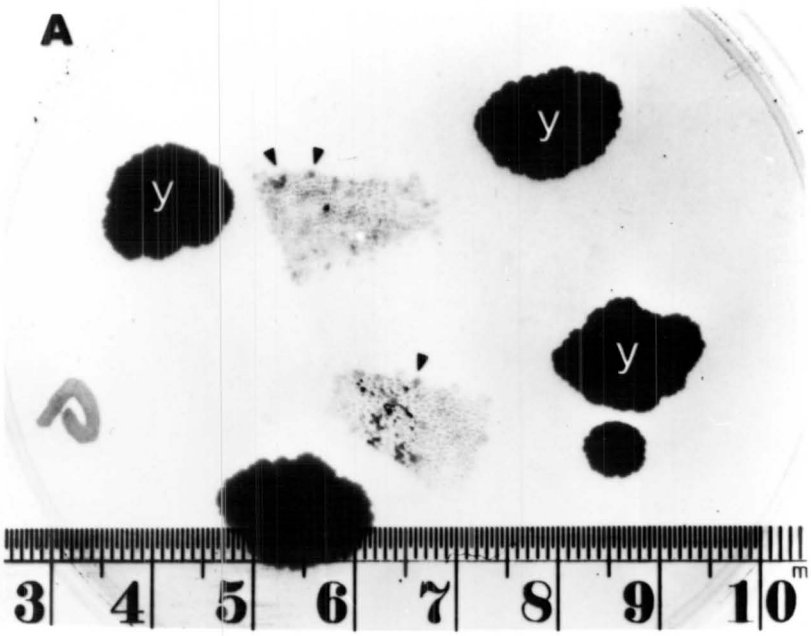


FIGURE 5.6 Effect of the yeast *Rhodotorula glutinane* on *S. luteus* spore germination.

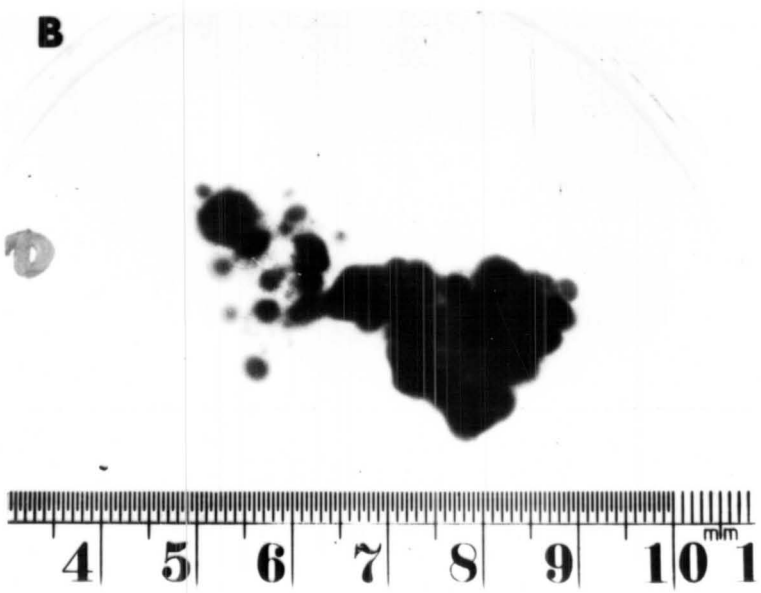
A Spores showing little germination (arrows), adjacent to yeast colonies (Y).

B Control plate exhibiting good spore germination on the nutrient medium alone.

5-6 A



B



5.3.3 Effects of Ultraviolet Light

Trione and Leach (1969) reported positive stimulation of sporogenesis in several genera of fungi by UV light and Tinline *et al.* (1960) have observed lethal and mutagenic effects of UV light on spore formation and growth of *Cochliobolus sativus*. In a preliminary study spore germination appeared to be stimulated by UV light during semi-aseptic synthesis investigations. Problems occurred with erratic seed germination and low infection levels from the spore inoculum. These were in part due to the low levels of spores used (5.0×10^4 spores/seed) and also to the use of vermiculite which was found to be excessively alkaline (pH 11.0). Where mycorrhizas formed, more infected short roots occurred, particularly as a result of short wave UV treatment.

Further studies, based on these observations, were carried out to determine the effects of UV light using the semi-aseptic synthesis technique. The materials and methods are discussed in Section 5.2 and the treatments used were as follows:

For both spore concentrations A (2.0×10^9) and B (5.0×10^7)

1. Control - no spores.
2. Spores - no treatment.
3. 2 minutes exposure to Long Wave UV.
4. 5 minutes exposure to LUV.
5. 2 minutes exposure to Short Wave UV.
6. 5 minutes exposure to SUV.

At the end of one month there were no mycorrhizal associations visible on the seedlings of any treatment as these had not yet produced secondary roots (2y). Their 'pioneer roots', mainly exploratory in nature (extending the rooting system), often bear infected dichotomous and non-dichotomous short roots, particularly near the basal region. After two months growth all seedlings, except those of the control, showed mycorrhizas. The fungus had in many cases, formed a sheath 1-2 cm along the 2y roots, as well as infecting short roots on the primary (1y) root. Uninfected dichotomous roots also occurred and these were morphologically different from infected roots. The uninfected roots were much longer, often up to 1 cm in length before dichotomy occurred (Fig. 5.7). Infected short roots appeared to be lacking in apical dominance. They were short and often hypertrophied even where the sheath was thin and they frequently lacked the pigmented epidermal layer of uninfected roots (Fig. 5.8). The extent of infection at this stage was confined mainly to the top two or three 2y roots - these would have emerged from the 1y root where spores had been deposited by pipette or dislodged from the seed coat during germination.

After four months, infection was quite extensive, particularly in the UV treatments. As well as the initial infection spreading along the 2y roots, there was evidence for 2y root tips becoming infected. This was probably caused by their coming into contact with other infected roots in the pot, since root growth is considered to be faster than that of fungal hyphae, although mycorrhizal mycelial strands

FIGURE 5.7 Uninfected dichotomous roots (U).

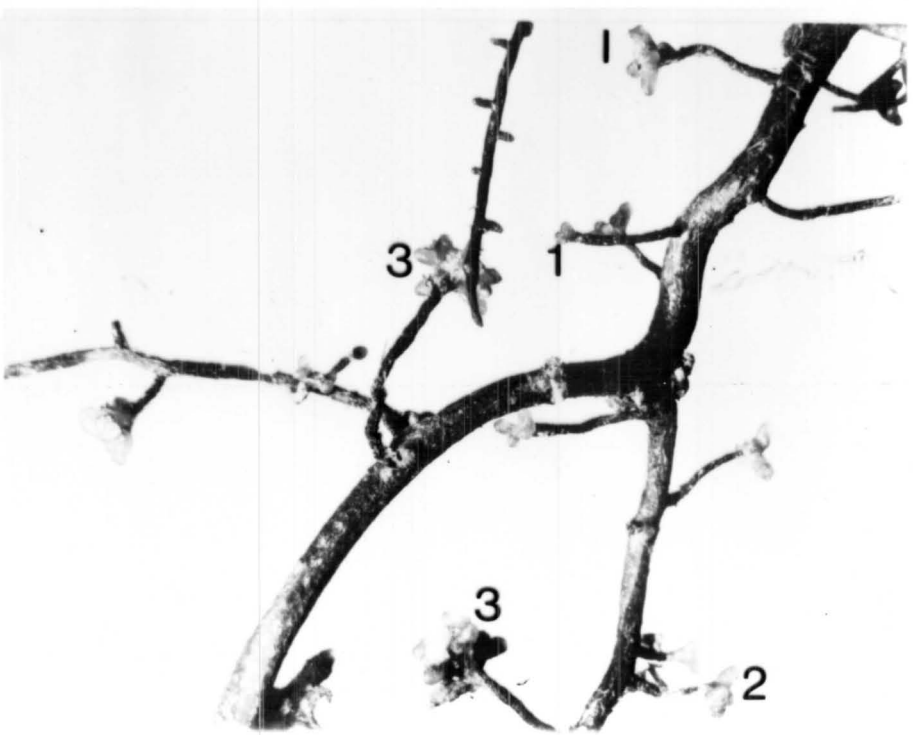
These are much longer and less swollen than infected roots (I) but are often found on the same root system. x10

FIGURE 5.8 Infected dichotomous roots (I).

A range of mycorrhizal roots is shown from those newly infected (1) through successive stages (2, 3) of multiple branching.
x15

5-7

5-8



grow 2-3 times faster than individual hyphae (Bowen and Rovira, 1976). Hyphae covered the tips of the infected 2y roots and formed a web over even the smallest root buds. The characteristic root hair zone was either non-existent in these cases or restricted in the number of root hairs (Hatch, 1937). The development of short roots behind the apex was very similar to that described by Robertson (1954) in P. sylvestris, consisting of an acropetal succession of short roots, the distal ones either simple buds or once forked (Fig. 5.9) and the proximal ones repeatedly dichotomised (Fig. 5.10) with a web of mycelial strands suspended between the short roots and over the surface of the 2y root (Fig. 5.11). These strands had extended some distance into the surrounding vermiculite but morphologically were not similar to rhizomorphs as described in Garrett (1970). These may develop at a later stage.

The numbers of mycorrhizal associations per treatment were counted and compared (Table 5.1). Each infected short root, showing dichotomy or not, was treated as one mycorrhizal association.

It is evident that some spore germination must have occurred between month 1 and 2, since all spore treated seedlings show mycorrhizas, but there was little difference between the treatments at this stage and infection still appeared to be rather erratic. After four months the majority of seedlings were showing mycorrhizas from the spore inoculum. The LUV treatments show no significant increase in numbers of mycorrhizas per seedling, but the SUV

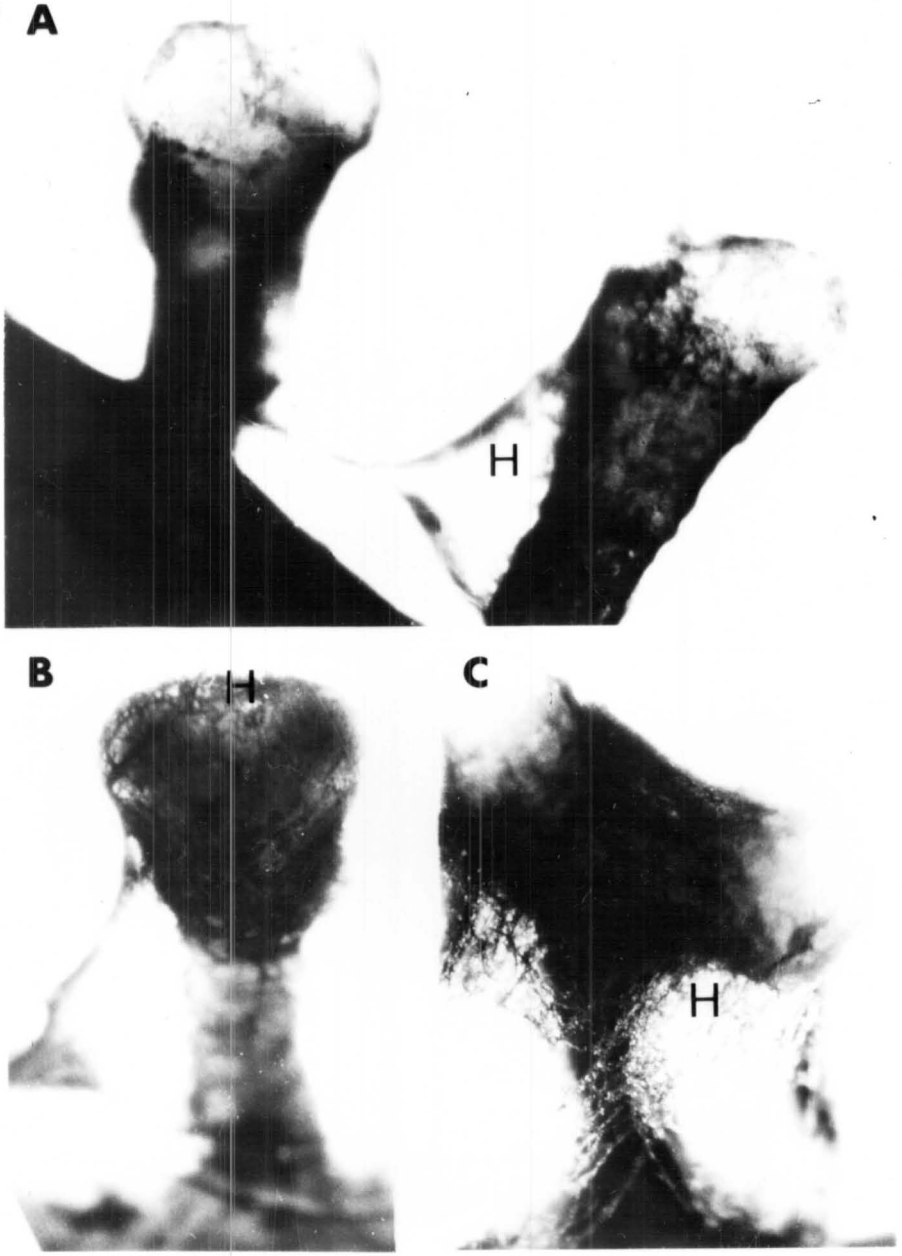
FIGURE 5.9 Simple dichotomous short roots.

Sequence of development of hyphal web (H) and root branching is shown in A, B and C. x60

FIGURE 5.10 Repeatedly dichotomised short roots.

All apical dominance appears to be lost allowing multiple branching to occur. Hyphae can be discerned on the root surface. x50

5-9 A



5-10

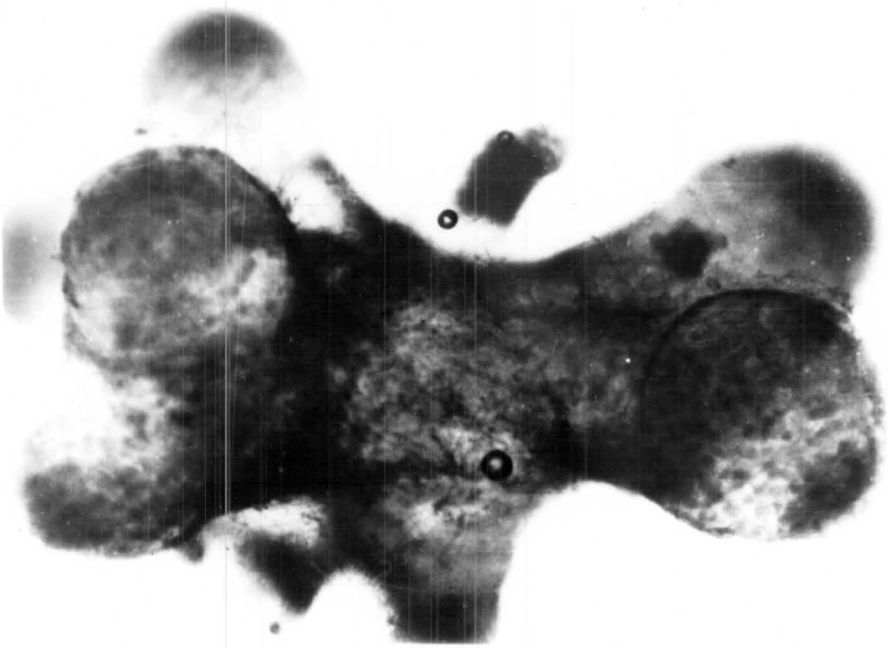


FIGURE 5.11 Mycelial strands.

A Seen on the surface of a young, mycorrhizal, root (arrows) (Lactophenol cotton blue stain). Note that the strands are extensive on the main root (R) between dichotomies (D).
x60

B Fine mycelial rhizomorphs seen on roots of a mycorrhizal seedling (arrows).
x35

5-11 **A**

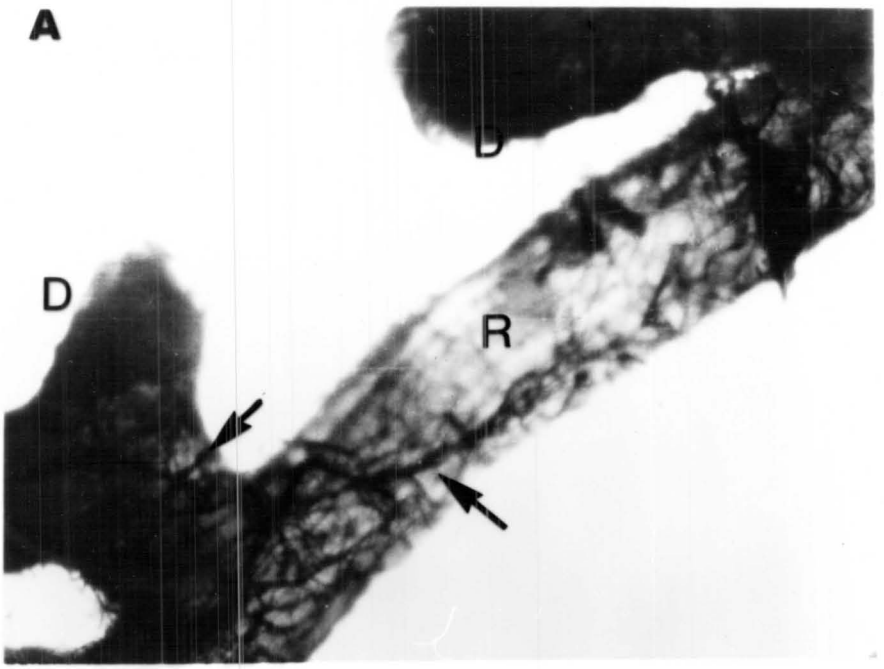


TABLE 5.1 EFFECT OF UV LIGHT ON MYCORRHIZAL INFECTION

Treatment	* 2 months after inoculation		4 months after inoculation		Treatment Means
	% infected seedlings	Mean No. infected roots/ seedling	% infected seedlings	Mean No. infected roots/ seedling	
Control	0	0	1	2.0	
1 Spores A	40	7.0	50	43.0	36.1 Cc
Spores B	10	16.0	40	29.2	
2 2 min LUV A	40	4.7	80	47.8	51.3 Bb
2 min LUV B	30	7.0	80	54.7	
5 min LUV A	30	5.0	100	50.2	50.6 Bb
5 min LUV B	20	7.5	100	51.0	
3 2 min SUV A	50	4.8	100	124.9	130.3 Aa
2 min SUV B	30	10.3	80	135.7	
5 min SUV A	60	15.0	100	152.7	140.4 Aa
5 min SUV B	30	8.0	80	128.1	
				(Std Error = $\pm$ 4.0)	

NOTE: * No associations after one month of growth.

1 A = Spore concentration of 2 x 10^9 spores/seed.
B = Slurry of spores on seed coat ($\simeq$5.0 x 10^7)

2 LUV = Long wave UV light treatment (254 mμ).

3 SUV = Short wave UV light treatment (365 mμ).

treatment has increased these by 2.5 - 3 times. The exposure to SUV has increased numbers of spores germinating but, from the results of the two month period, it seems likely that enhanced infection only becomes effective after considerable time has elapsed. By the end of two months the first foliage leaves of the seedlings have set, this being generally acknowledged as the time at which mycorrhizal infection actively begins (Huberman, 1940; Robertson, 1954; Harley, 1948; Wilson, 1951, Boullard, 1960; Laiho and Mikola, 1964) if the inoculum is also active and capable of infecting the roots. Infection is dependent on spore germination and subsequent mycelial growth when using a basidiospore inoculum. It would appear that spore germination was enhanced by SUV light and also fungal isolations made from these roots grew very actively compared with other pure cultures. Morphologically these cultures were identical. Little difference in infection was shown between the two spore concentrations used. In the damp vermiculite, spores coated on the seeds could be easily dislodged and not carried away on the testa by the developing epicotyl, often a problem with inocula dried on the seed. Similarly there was little difference between the 2 or 5 minute UV treatments used.

5.3.4 Effects of Chemical Stimulants

Many chemical stimulants have been implicated in fungal spore germination (Sussman and Halvorson, 1966; Weber and Hess, 1976; Bowen and Theodorou, 1973). Problems were encountered when using stored spores, therefore this

experiment was set up using spores dropped directly from hymenial tissue onto agar plates. To overcome the variable germination rates in different sporophores each plate had a random sample of tissue from three of the several sporophores used. Five replicate plates per treatment were seeded with spores from 15 pieces of hymenial tissue, the tissue being removed after this. During the incubation period, plates were observed and numbers of germinating spores in each field of view counted. The spore drops vary in the number of spores they contain and only areas of each drop zone containing approximately 500 spores/mm^2 ($\pm$ 10 percent) at 100x magnification were chosen for observation. For each treatment, 60 counts at random within areas of the above density were made and the total number of spores germinating per field of view recorded. At 100x magnification the radius of view = 0.95 mm therefore the area of observation = $\pi \times 0.95^2 = 2.8353\ mm^2$. Since 1 mm^2 contains approximately 500 spores, 2.8353 mm^2 contains about 1,418 spores in the field of view. The figures given in Table 5.2 are expressed as a percentage of this. The treatments used were as follows:

1	Benzaldehyde	Some plates left 1 hour, others overnight in saturated atmosphere of these volatiles.
2	Acetone	
3	Ethyl acetate	
4	Chloroform	
5	1, 2 Dichoroethane	
6	Furfuraldehyde	
7	Inositol	0.1 g/l in M40 agar
8	Oxo-glutaric acid	0.05 g/l in M40 agar

9	Malic acid	0.05 g/l in M40 agar
10	Adenine HCl	0.05 g/l in M40 agar
11	Nicotine acid	0.05 g/l in M40 agar
12	Thiamine HCl	0.05 g/l in M40 agar
13	Folic acid	0.05 g/l in M40 agar
14	Pimelic acid	0.05 g/l in M40 agar
15	Fumaric acid	0.05 g/l in M40 agar
16	Shikimic acid	0.05 g/l in M40 agar
17	Citric acid	0.05 g/l in M40 agar
18	Succinic acid	0.05 g/l in M40 agar
19	Dimethylaminobenzaldehyde	0.05 g/l in M40 agar
20	Coumarin	0.01 g/l in M40 agar
21	NAA	0.01 g/l in M40 agar
22	Kinetin	0.005 g/l in M40 agar
23	$CaHPO_4$	0.5 g/l in M40 agar
24	Ca Phytate	0.5 g/l in M40 agar
25	EDTA	0.5 g/l in M40 agar
26	Malt Extract	5.0 g/l in M40 agar
27	Casein Hydrolysate	5.0 g/l in M40 agar

Solutions of treatments 7-27 were all millipore filtered and added to agar prior to pouring the plates. The control used was M40 A agar without the thiamine supplement and the results after three weeks incubation at 25°C are given in Table 5.2.

TABLE 5.2 EFFECT OF CHEMICAL STIMULANTS ON BASIDIOSPORE GERMINATION

Treatment	% Germination ≥0.1%	Treatment	% Germination <0.1%
Nicotinic Acid	0.96	NAA	0.06
Inositol	0.85	$CaHPO_4$	0.06
Citric Acid	0.19	Adenine HCl	0.03
Malt Extract	0.16	Fumaric Acid	0.03
Control	0.00	EDTA	0.02
		Thiamine	0.02
		Pimelic Acid	0.02

In the case of nicotinic acid and inositol plates it was noted that some of the spore drops were a mass of mycelial growth and the counts were limited to those not obscured by mycelium. The figures given are probably very conservative because spores were observed to be germinating on these plates after only 1-2 weeks. The plates were kept for about eight weeks and eventually germination on the controls rose above 0.01% but by this stage mycelial growth on most other plates was extensive. Apart from coumarin, succinic acid and Ca phytate (all showing about 0.01% germination) none of the other treatments stimulated spore germination. The overall figures for germination *in vitro* are still low, considering numbers of spores evidently viable (see Chapter VI), but both nicotinic acid and inositol

appear to considerably increase germination.

5.3.5 Discussion

According to Gregory (1966) the basidiospores of S. luteus would be classed as xenospores (those becoming detached and dispersed) and they generally exhibit exogenous dormancy. Internally the cytoplasm is surrounded by an invaginated plasmalemma (Stocks and Hess, 1970; Heintz and Niederpruem, 1971; Hawker and Madelin, 1976) and contains endoplasmic reticulum, various vesicles and mitochondria. Microbodies, often closely associated with the endoplasmic reticulum, have also been observed (McLaughlin, 1973; Maxwell et al. 1977). Storage bodies containing lipids, glycogen granules and membrance bounded storage vacuoles for phospholipids are very frequent. The pre-germination spore wall generally has 1-3 layers containing variously hemicelluloses, chitin, pectic materials, proteins, lipid and melanin (Hawker and Madelin, 1976; Rast and Hollenstein, 1977). The wall ultrastructure as determined by Rast and Hollenstein (1977) in Agaricus bisporus spores is composed of an outer layer of melanin rich, amorphous, material interspersed with some chitin, a median layer of chitin fibrils embedded in a β-glucan-protein matrix and an inner layer of mucous. Lipid is found throughout the wall. They found that the outer layer is highly resistant to attack by 6 N HCl, but the other layers are removed. Similar features have been shown for S. luteus spores (pers. comm. L.G. Greenfield), indicating that high levels of chitin were present (approximately 25 percent) as measured by the amount of hexosamine (42 percent of total N)

and that the integrity of the wall structure was maintained during this treatment as observed in S.E.M. preparations of hydrolysed spores. It has also been shown that the inner wall layers form the plug to which the sterigmata was originally attached (Stocks and Hess, 1970; Heintz and Niederpruem, 1971; Olāh, 1973; Ginns and Kokko, 1975; Akai et al. 1976; Rast and Hollenstein, 1977). The basidiospore, therefore, has a relatively resistant ectospore layer and a weaker plug consisting of epi- or endospore material through which the germ-tube may emerge.

The most obvious change occurring during germination is swelling of the spore caused by water imbibition and this would initially appear to involve uniform cell wall growth followed by polarized growth centred on a small area of the cell wall (Cochrane, 1958; Smith et al. 1976). During this time the cell wall layers may alter considerably and the germ-tube emerges from the germ pore, its wall layers being continuous with those of the endospore (Stocks and Hess, 1970; Heintz and Niederpruem, 1971; Smith et al. 1976). This swelling was obvious in S. luteus spores and the polarized growth tended to be centralized on the lateral walls. The changes seen within the spore cytoplasm are probably related to changes in droplets and vacuoles containg lipids as these increase in size during germination (Heintz and Niederpruem, 1971; Hess and Weber, 1976). Other ultrastructural changes known to occur at germination include a marked increase in endoplasmic reticulum and ribosome number and in the size and number of mitochondria. Related to cell wall synthesis

there are increases in numbers of cytoplasmic vesicles (see Bartnicki - Garcia, 1973) and lomasomes (Smith et al. 1976).

The poor germination of many spores when densely crowded on a surface can be caused by self-inhibition (Cochrane, 1958, 1974, Macko et al. 1976) and is only overcome by favourable conditions for growth. Under natural conditions the greater the number of spores in the vicinity of a seedling, the better the chance it has of developing mycorrhizas which still increase to about 10^8 spores/seed. In vitro studies, however, show much lower levels of density tolerance for spore germination (about 10^2 - 10^3 spores/mm^2 on laboratory media).

The Embden-Meyerhoff-Parnas (EMP) pathway is considered to be present in all spores and under aerobic conditions is probably producing acetyl-Co A from glucose to be oxidised in the Citric acid cycle (CAC). Carbon dioxide is not produced under anaerobic conditions thus the system may not be functional, or it may accumulate pyruvate and lactate until an inhibitor is removed. Both the CAC and the terminal electron transport (TET) systems appear to be intact in spores although some enzymes are absent in ungerminated spores but with the onset of germination protein synthesis increases up to four fold and the total respiratory enzyme activity may increase 2 - 210 times (Gottlieb, 1976). Oxygen consumption during germination is high and is stimulated by additions of sucrose, some hexoses and xyloses, but not glucose. The hexose monophosphate (HMP)

shunt is involved with the initial oxidation of glucose in Schizophyllum spores, but the EMP pathway becomes increasingly more important as germination proceeds (Hess and Weber, 1976). The lipids are not only important as energy sources for respiration, but also are found to be incorporated into amino acids, organic acids, carbohydrates and in particular the germ-tube wall (Reisener, 1976).

In relation to the chemical stimulants used in this study, the preference of basidiomycetes for ammonium N is again apparent. It is probable that the major and primary reaction of ammonium assimilation is the formation of glutamic acid which is transaminated to amino acids. This would account for the stimulated mycelial growth noted after spore germination. Nicotinic acid is a constituent of the respiration coenzymes diphosphopyridine nucleotide and triphosphopyridine nucleotide and as such may trigger off the breakdown of carbohydrates by the HMP pathway, thus initiating germination. It is also related to the synthesis of glutamic acid since this requires carbon and energy from glucose fermentation, a process known to require nicotinic acid, thiamine and possibly inositol (Hawker, 1950). By reaction with glutamine (derived by the interaction of glutamic acid with ATP and ammonium) nicotinic acid plus more ATP therefore gives DPN. Glutamine is also involved in other diverse synthetic pathways, eg those of hexosamines, purines and tryptophan (White et al. 1968). The involvement of inositol is somewhat more difficult to understand as no general function is ascribed to it. The comparatively large amount required by most fungi indicates

that it may function as an accessory food-factor rather than part of an enzyme or coenzyme. It is specific in its action, overcoming inhibition caused by high levels of some other vitamins, particularly thiamine and biotin (Hawker, 1950; Lilly and Barnett, 1951; Cochrane, 1958). In its active stereoisomeric form (myo-inositol) it is known to be a constituent of certain phospholipids (the phosphoinositides) and as such may be a structural component of the spore (Cochrane, 1958; White et al. 1968; Smith and Berry, 1974). The vitamins aneurine (thiamine) and biotin (Vitamin H) are also reported germination stimulants (Harley and Lewis, 1969; Rovira and Harris, 1961; Robbins, 1952).

Stimulation of germination by citric acid may be due to derepression of enzyme systems of the TCA cycle and that by malt extract could be caused by components rendered inactive by autoclaving in normal sterilisation of M.E. type media, but retaining their activity when millipore filtered.

The effects of visible light have been implicated in spore germination (Cochrane, 1958) although the involvements of UV light has not been studied except for its known influences on sporogenisis, mutability and lethality of micro-organisms. The wavelengths that are most effective in these processes range from 250-280 mμ in fungi and lethality occurs about the critical absorption wavelength for nucleoproteins (<240 mμ). The response of the spores is also dependent on the degree of melanisation of the spore walls. Pigmentation makes them less susceptible to radiation damage and may prevent lethal or mutagenic effects

from occurring even when the radiation is in such a range (Sussman and Halvorson, 1966).

NOTE IN PROOF

A recent paper by N. Fries (Basidiospore germination in some mycorrhiza forming hymenomycetes - Trans. Brit. Mycol. Soc. 70, 319-, 1978) has indicated the difficulties of germinating basidiospores on laboratory media. Using spores from Laccaria laccata, Amanita muscaria, Lactarius helvus, Paxillus involutus and Leccinium scabrum slight germination (generally $\leq$0.01 percent) only occurred when activated charcoal and Rhodotorula glutinis were combined on test plates. The yeast was previously reported to induce spore germination in Suillus (Fries, 1941). It is noted that spores were transferred to plates in a distilled water suspension, a process shown to inhibit spore germination in this study. Ammonium ions are mentioned as germination inhibitors of Suillus spores but in this investigation permitted germination of the spores.

CHAPTER VI

BASIDIOSPORE STORAGE AND VIABILITY

6.1 INTRODUCTION

The methods commonly used in spore storage programmes consist of conventional means of preserving microbial tissue - cold storage or freeze drying - but even with these treatments, most basidiospores from mycorrhizal fungi appear to have inherently low viability. Most studies utilising introduction of spores to plants have found it necessary to use high spore counts (usually $>10^6$ spores per seed) to overcome the low incidence of germination. This may, however, introduce problems of spore crowding and consequent exogenous dormancy (see Chapter V). Competition of the germinating spores with other soil micro-organisms also has a detrimental effect on mycorrhizal formation, as shown by Theodorou (1967, 1971) and Marx and Bryan (1975) in studies with fumigated or sterilised soils. Availability of suitable storage procedures to maintain viability germination is considered an essential part of inoculation programmes since the stimulation of germination by chemicals is still in its infancy and little is known about fungal nutritional requirements for production of sporophores in pure culture.

6.2 MATERIALS AND METHODS

Spores were collected from spore drops on glass plates and stored in dated Bijou bottles for subsequent use. All cool stored spores were kept refrigerated at approximately 5°C, some being kept in a closed container with anhydrous $CuSO_4$ to maintain a dry atmosphere. Frozen spores were kept in the freezer compartment of the same unit at -2°C. Freeze dried spores were quick frozen by immersion in liquid nitrogen and vacuum dried in an Edwards high vacuum centrifugal freeze drier (Model 30 P2/822) at 0.1 Torr. Sporophore hymenial tissue for use as inoculum was also prepared in a similar manner. After vacuum drying the tissue was passed through a series of analytical soil sieves to give particle sizes of <0.5 mm and <0.2 mm (for use in seed coating studies) and kept at room temperature in a dessicator. Some spores were also kept at room temperature in sealed Bijou bottles.

Spore viability was studied using vital and other stains as described by Gray (1954) and Gurr (1960, 1965) and by the acridine orange method of Stack and Sinclair (1975). All stains were used at 0.1 percent concentrations in aqueous solutions where applicable. After staining with acridine orange, spores were observed by fluorescent microscopy. The source of UV light was provided by a mercury vapour lamp. BG 38 (4 mm) and BG 12 (3 mm) exciting filters were used in conjunction with a K510 or K530 suppression filter to obtain the correct fluorescence. Dead spores and organic matter fluoresce shades of orange or brown and live spores appear

green in colour. Kodak HP4 film was used for black and white photography and HS Ektachrome (160 ASA, daylight) for colour photography. The percentage of surviving spores was determined by counting numbers of non-fluorescing spores as opposed to total numbers of spores on photographic plates.

A glasshouse experiment was set up using semi-aseptic synthesis methods to test the efficiency of the stored spores to initiate mycorrhizas on pine seedlings. Soil was transported from a site near Broken River having no vegetation cover and would correspond to the $A_{1/2}$ - AB horizon of the Puketeraki Soil (Chapter IV, Section 3.5). The soil was passed through a 1 x 1 cm sieve to remove rocky waste, which was replaced with fine gravel, and then sterilised in a Camplex soil steriliser (4 hours after stabilising at 85°C) and stored in sealed polypropylene bags. Seeds of P. mugo (FRES 74/177) were treated as follows before being planted in 15 cm diameter pots.

A Seeds surface sterilised in 10 vol H_2O_2 (2 hours) and washed in sterile distilled water.

B Seeds soaked 24 hours in distilled water at 4°C, surface sterilised as in A.

C Seeds dusted with thiram (seeds were placed in a bag with thiram powder and shaken until liberally coated with the fungicide).

The inoculum sources were:

1	Freeze dried spores	A	1975
		B	1976
		C	1977
2	Cool stored spores	A	1977
		B	1977 (over dessicant)
3	Frozen spores	A	1975
		B	1976
		C	1977
4	Freeze dried hymenial tissue (1977)		
5	Control (spore carrier only)		
6	Pure culture of S. luteus mycelium.		

Spores from inoculum sources 1, 2 and 3 were added in 0.5 g amounts to 250 cc of a sterilised sand/vermiculite 'carrier' (50/50 v/v) and mixed thoroughly. Each of the five replicates/treatment received 50 cc of the spore/'carrier' mix, this being equivalent to 4.0×10^8 spores in each pot (0.1 g spores contains approximately 4.0×10^8 spores as previously determined by haemocytometer counts). For inoculum source 4, 2.5 g of hymenial tissue/250 cc of 'carrier' was used (since 0.1 g of this contains approximately 5.3×10^7 spores, many of which are immature, each replicate received about 2.6×10^8 spores). Inoculum source 6

received 25 cc of mycelium plus 25 cc of 'carrier' per replicate. A peat/vermiculite mixture (moistened with 200 ml of medium G (Appendix 1) per 850 cc of the mixture) was used to grow the pure culture inoculum. The mixture was previously inoculated from M40 agar cultures of S. luteus and grown for six months at 25°C.

These inoculum 'carriers' were well mixed with the top 1-2 cm of sterile soil and five seeds were planted per pot, being thinned to three seeds/pot after three months. The experiment was set up under glasshouse conditions and although adverse conditions with dull days and low temperatures (average minimum temperature $\simeq$9°C, average maximum temperature 18°C over a period of two and a half months) initially retarded seedling growth, they were assessed after six months for mycorrhizal formation. Few differences in size of the seedlings were evident at this stage because of the slow growth rate of P. mugo, therefore dry weights were not determined.

6.3 RESULTS AND DISCUSSION

6.3.1 Staining of Basidiospores

The vital stains methylene blue and neutral red were initially used in an attempt to determine stored spore viability. Changes observed in the spores were as follows:

A Spores stored at room temperature (young and old) had dark staining cytoplasms containing

granular bodies and some vacuolate areas. Those stored at $4^{o}C$ were similar in appearance. Morphologically they were identical to those spores discussed in Chapter V (see Fig. 5.3).

B Freeze dried spores were generally of similar appearance to those of A, although some showed minor cytoplasmic condensation.

C Frozen spores stained only lightly and 1-2 percent had ruptured spore coats.

D Homogenised, frozen fruiting body was obtained from FRES, however, the spores in this material showed extreme condensation of the cytoplasm, leaving large vacuolate spaces and were more darkly stained (Fig. 6.1). Large numbers of bacteria were present in these samples.

E Spores humidified overnight in a moist chamber were similar to A and B in appearance but stained slightly darker.

Differences could clearly be seen with these two stains, but could also be detected without staining. A number of other stains were investigated to see if viable spores could be easily distinguished. These are set out in Table 6.1 in order of decreasing ability to show differentiation of spores.

6-1

FRES. INOCULUM SPORES.

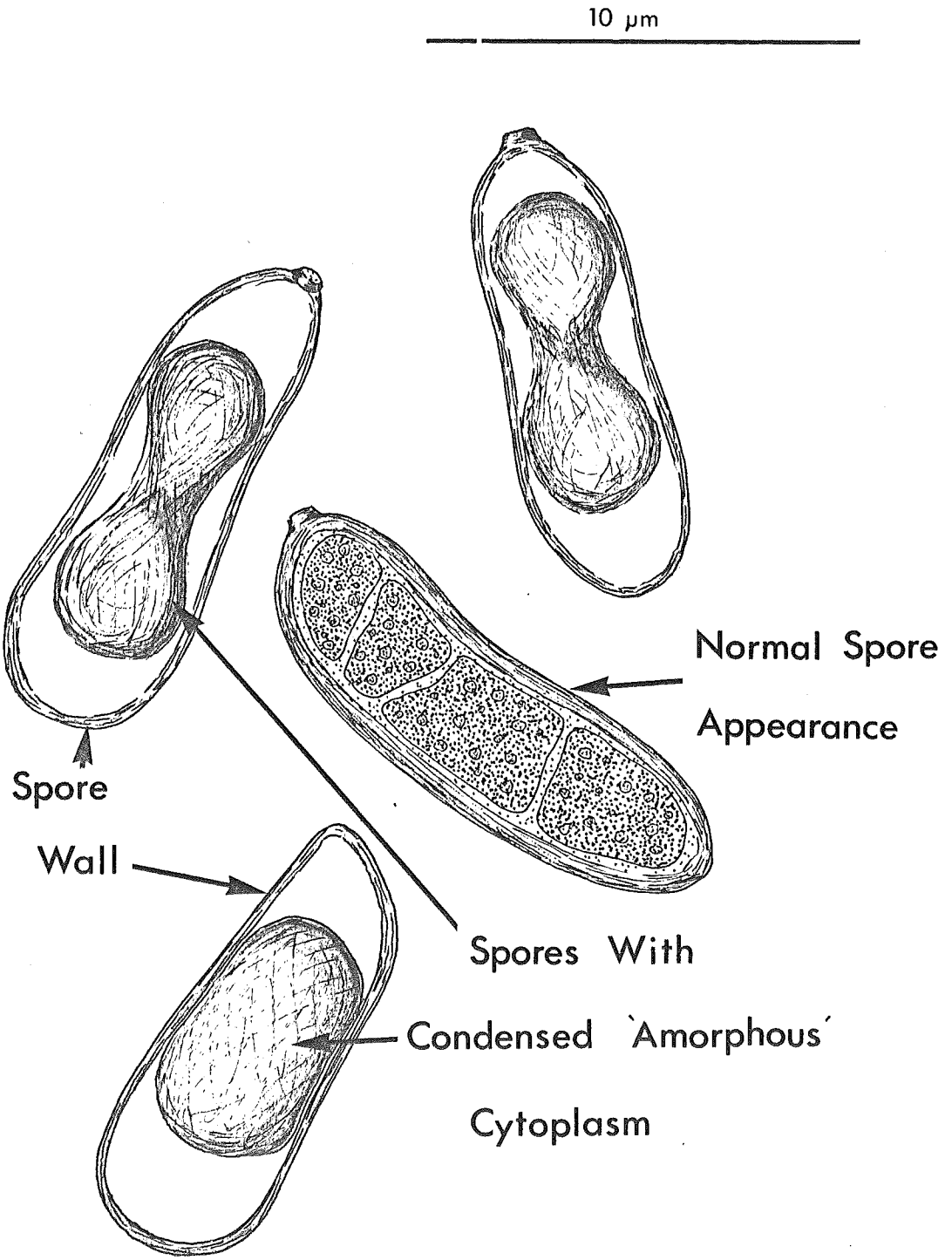


TABLE 6.1 SPORE STAINS

Stain (Solvent)	Results
Chlorazol Black E (Aq)	Vital stain showing some spores much darker than others, some very swollen as if germinating.
Basic Fuschin (Aq), Schiffs	Some spores deep mauve colour in cytoplasm but most a light green. Empty spores clearly defined.
Aniline Blue (Aq - acidified)	Most spores blue-green, some showing a deep blue cytoplasm. Empty spores light green.
Ruthenium Red (Aq)	Most light red, some with dark red cytoplasm. Empty spores outlined in red.
Rhodamine B (Cellulose)	Vital - some dark red, most light and empty spores outlined in red.
Toluidine Blue (Aq)	Most blue-green, some dark green.
Lacmoid (Aq)	Most mauve, some dark purple, empty spores obvious.
Trypan Blue (Aq)	Some dark blue, most bluish.
Trypan Blue (Lactophenol)	Empty spores very obvious.
Azur B (Aq)	Most light blue, some dark blue.
Safranin (Aq)	Most pink-red, some dark red.
Thionine (Aq)	Vital - most blue, some deep purple.
Malachite Green (Aq)	Most green, some dark green.
Ethyl Violet (Aq)	All spores light red except empty ones - dark red.
Bromophenol Blue (Aq)	All light red, same effect on empty spores.
Aniline Green (Aq)	All spores light green, some cytoplasms with blue tinges.

Stain (Solvent)	Remarks
Primuline (Cellosolve)	Little effect but metachromatic granules evident in some spores.
Euchrysin 2 (Aq)	Cytoplasm uniformly green.
Fast Green (Aq)	Cytoplasm blue-green, no differentiation.
Brilliant Blue FCF (Aq)	Vital. Cytoplasm uniformly blue-green, empty spores conspicuous.
Nigrosin (Aq)	Empty spores stain dark blue.
Alizarin Red 5 (Aq)	All uniformly red.
Erythrosin B (Aq)	All uniformly red.
Crystal Violet (Aq)	All blue-purple in colour.
Magdala Red (Aq)	All red in colour.
Indigo Carmine (Aq)	All deep blue.
Lissamine Green (Cellosolve)	Uniformly light green.
Sudan Black (Ethanol)	All blue-black in colour.

Where spores were differentiated by these stains, their viability was still uncertain as the stored spores used could not be germinated *in vitro*. Use of the acridine orange technique outlined by Stack and Sinclair (1975) proved to be superior in differentiating between dead and live spores as can be seen in Fig. 6.2. Numbers of spores not fluorescing were determined by counting and the percentage viable are shown in Table 6.2

FIGURE 6.2 Acridine orange staining of stored spores (fluorescent microscopy).

A Frozen spores

B Cool stored spores

C Freeze dried spores

All spores fluorescing (bright) are considered to be dead. x300

6-2

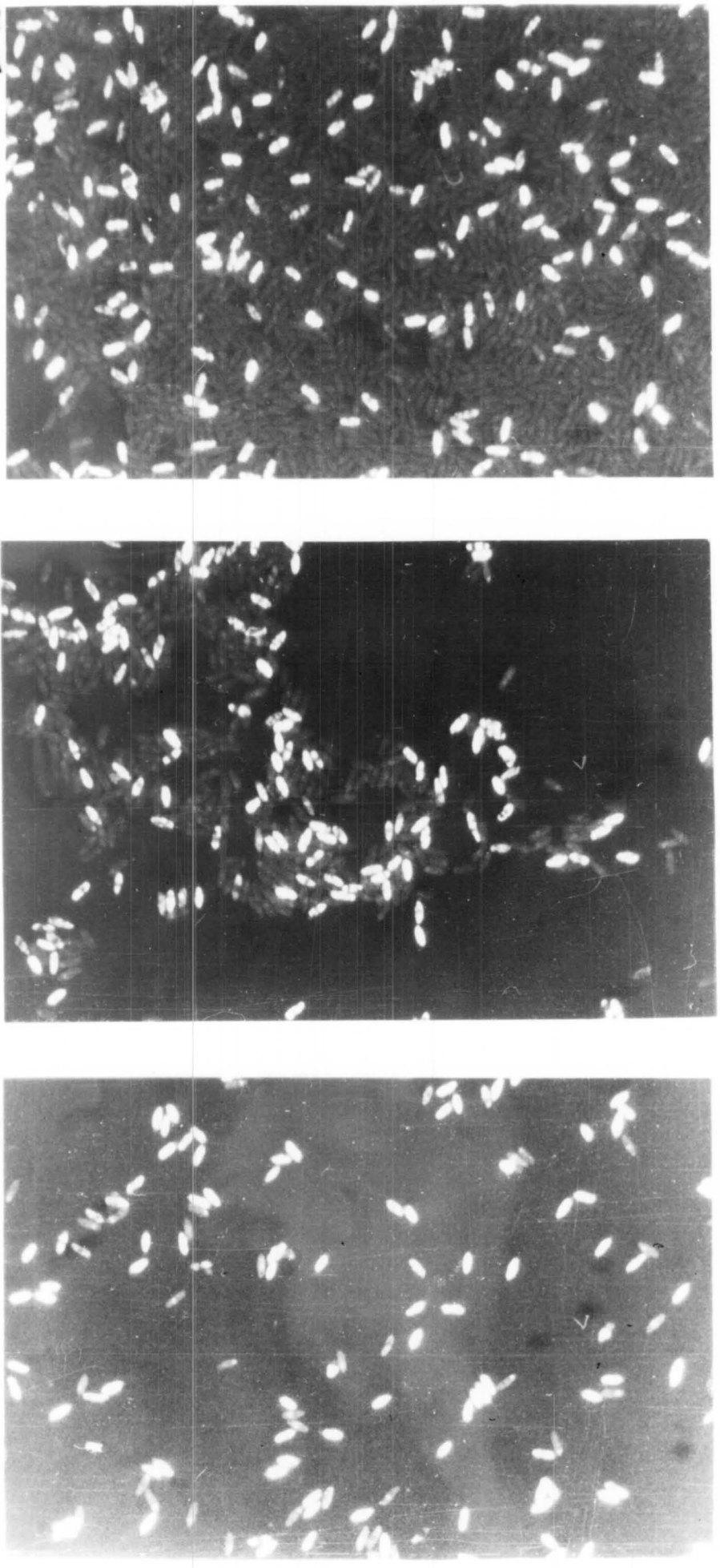


TABLE 6.2 SPORE VIABILITY ASSESSED BY ACRIDINE ORANGE STAINING

Treatment	Frozen Spores	Cool Stored Spores	Freeze Dried Hymenium	Freeze Dried Spores
Percent viable spores (non-fluorescent)	67.0	49.0	12.0	5.0

To obtain satisfactory numbers of spores for counting, photographs were taken at 100x magnification. Colour film was used (Fig. 6.3) but it was not possible to determine numbers of non-fluorescing spores because of the dark background produced by the UV light. The negatives were therefore re-exposed on to ASA 400 film and comparative counts were made from these.

The results indicate that frozen or cool stored spores have retained a medium level of viability during storage and would presumably be suitable for inoculation purposes. To test the applicability of this method of determining spore viability, seedlings were inoculated with the stored spores using the semi-aseptic synthesis technique described in Section 6.2

6.3.2 Spore Viability Indicated by Mycorrhiza Formation

The production of mycorrhizas on seedlings by the

FIGURE 6.3 Stored spores stained with acridine orange and photographed by fluorescence microscopy. Orange spores are dead; dark ones are alive.

A Frozen spores.

B Cool stored spores.

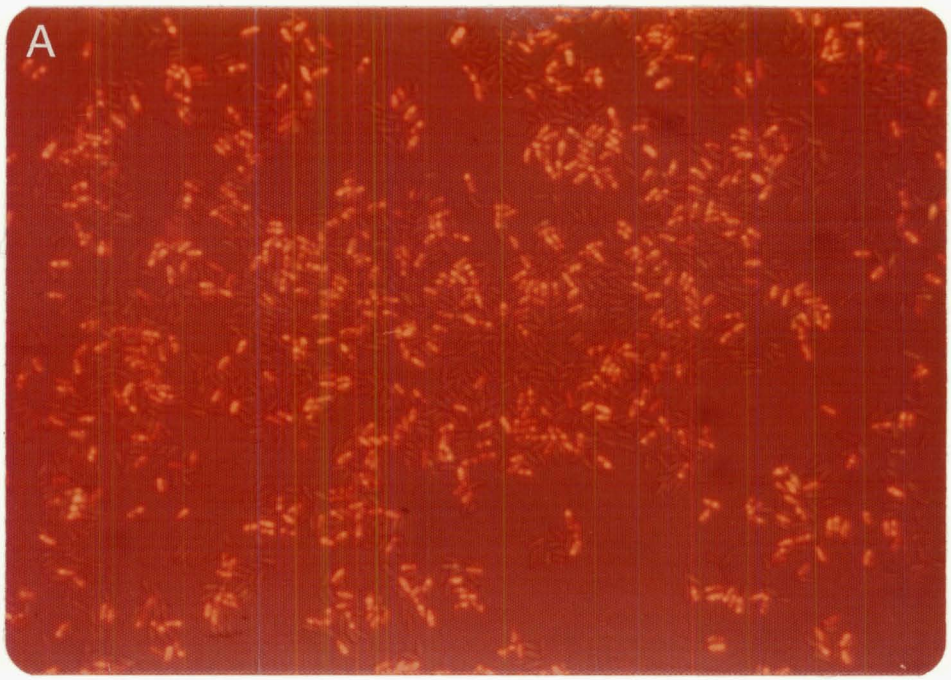
C Freeze dried hymenial tissue.

D Freeze dried spores.

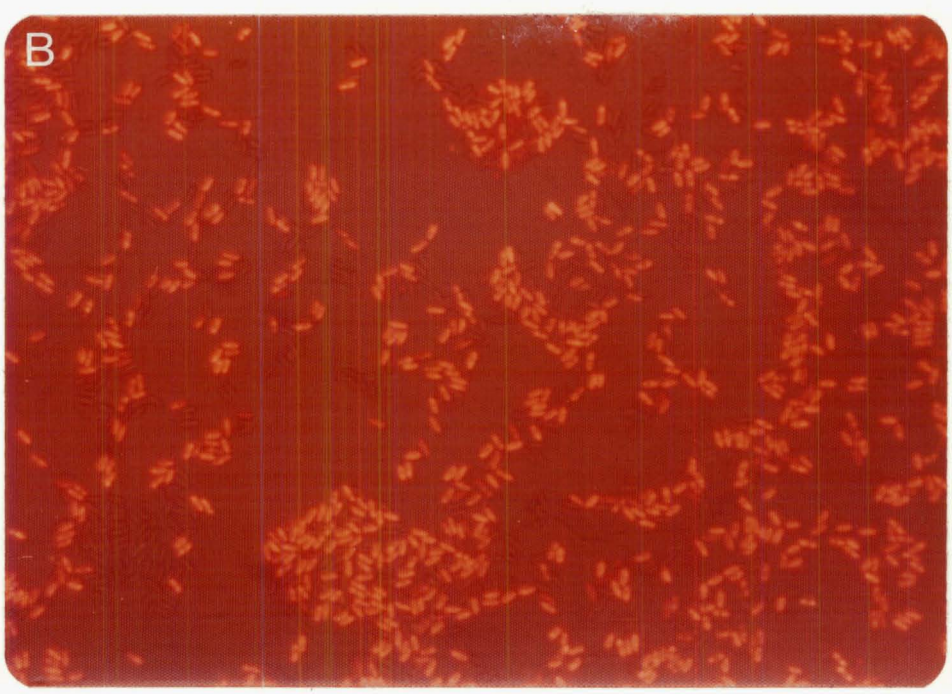
overleaf

All x250

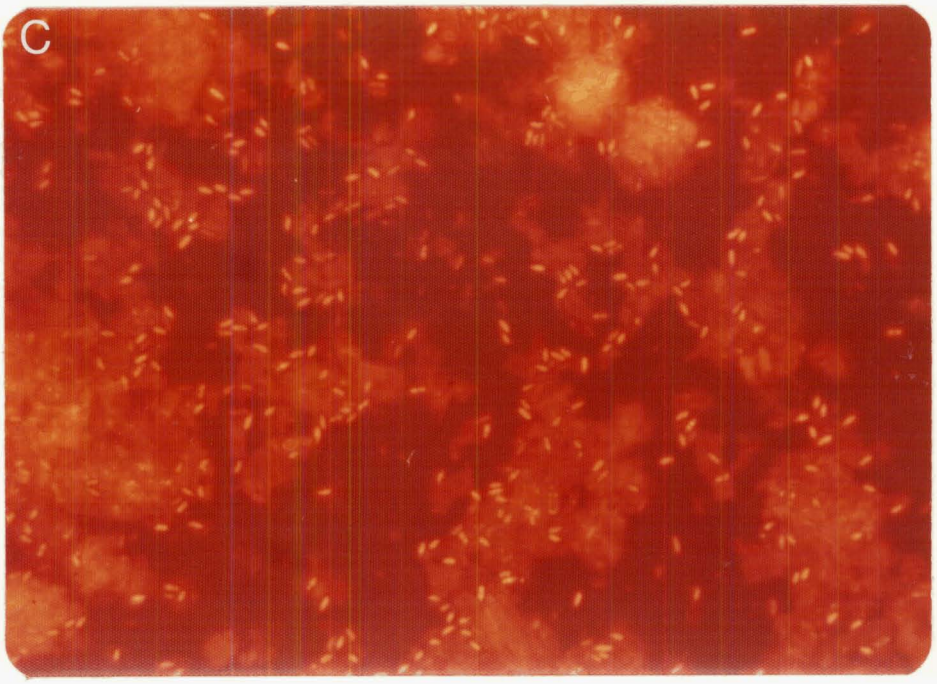
6-3 A



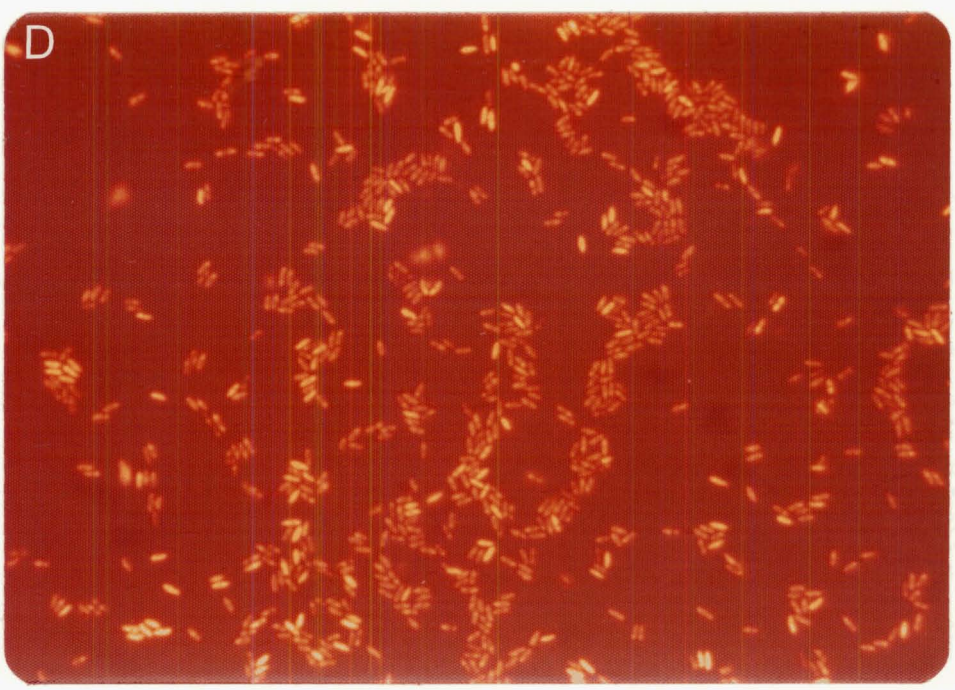
B



6-3 C



D



variously stored spores is summarised in Table 6.3. Considering the seed treatments, it is evident that there was little difference between the surface sterilised or soaked seeds in the formation of mycorrhizas. The coating of seeds with thiram had detrimental effects on seed germination (see also Chapter VII, Section 3.4). Very few seedlings grew and those that did had reduced numbers of mycorrhizas on their roots. As the effect of thiram was obvious data for the other seed treatments was analysed separately.

Considerable differences were seen between the various inoculum sources used. The best sources were cool stored spores (over dessicant) and frozen and cool stored (1977) spores were ranked next. Freeze dried spore and hymenial tissue inoculum also gave good mycorrhiza formation, but that of the pure culture was poor. Significant yearly decreases in the ability of both freeze dried and frozen spores to form mycorrhizas were evident.

6.3.3 Discussion

A number of vital and other stains were observed for indication of spore viability but the only one showing promise was acridine orange. From results of semi-aseptic mycorrhizal synthesis experiments it was found that spore staining may not be giving a correct indication of those surviving since the spores showing greatest viability by staining did not necessarily produce the greatest numbers of mycorrhizas. Spore viability indicated by this method must,

TABLE 6.3 EFFECT OF SPORE STORAGE ON MYCORRHIZA FORMATION

(Analysis of Variance of Mycorrhizal Means)

Seed Treatments			INOCULUM SOURCES									
			Freeze Dried Spores			Cool Stored Spores		Frozen Spores			Freeze Dried Hymenium	Pure Culture	Seed Treatment (Means of A)
			1975	1976	1977	1977	1977 (dessicated)	1975	1976	1977	1977	1977	
	Surface Sterilised Seeds	A	7.8	20.6	22.2	38.7	42.2	7.8	12.3	36.8	24.9	12.8	22.5 Zz
		B	73	100	100	100	100	100	100	100	100	100	
	Soaked Seeds	A	6.7	16.7	30.5	35.9	39.1	6.9	12.7	36.7	20.3	11.4	21.7 Zz
		B	73	100	100	100	100	100	100	100	100	100	
	Inoculum Source (Means of A)		7.3 Gg	18.6 Ee	25.6 Cc	37.3 Bb	40.6 Aa	7.4 Gg	12.5 Ff	36.8 Bb	22.6 Dd	12.1 Ff	Std Error = ± 0.4
	Thiram Dusted Seeds	A	7.2	12.5	14.5	12.2	13.9	5.3	10.4	19.2	9.4	10.6	
		B	33	66	60	90	83	100	100	100	100	100	
		C	(6)	(3)	(5)	(10)	(6)	(6)	(2)	(4)	(2)	(2)	

A % mycorrhizas on infected seedlings.
B % of assessed seedlings showing mycorrhizal infection
C Number of surviving seedlings.
Control seedlings = 0% mycorrhizas.

therefore, be assessed cautiously. Cool stored spores and frozen spores appeared to be the best inocula but spore viability decreases considerably with extended storage periods. It is essential, therefore, to use fresh inoculum sources to provide adequate mycorrhizal infection. The use of antifungal agents, such as thiram, dusted on seeds used for revegetation work is not recommended as germination may be adversely affected and establishment of seedlings impaired.

CHAPTER VII

MYCORRHIZAL INOCULATION OF SEEDS

7.1 INTRODUCTION

Many of the problems associated with establishment and growth of trees used for high altitude revegetation could be overcome if the myco-symbiont is introduced with the seeds to reafforestation sites. Selected inoculum sources would ensure the establishment of effective mycorrhizal associations and consequent rapid seedling growth. The use of basidiospores as the inoculum would prelude bulk problems associated with inocula such as mycorrhizal duff or pure cultures of the fungi. Little work has been done to develop a basidiospore inoculum suitable for coating seeds. Low spore germination levels and problems with spore viability after storage have prevented use of them as an effective inoculum source. The concentration of spores necessary for effective mycorrhiza formation is generally high, ranging from 10^6 spores/seed to in excess of 10^9 spores/seed depending on spore storage conditions (Marx and Ross, 1970; Theodorou, 1971; Theodorou and Bowen, 1973; Marx, 1976). If greater quantities of spores are used, high spore densities (see Chapter V, Section 3) may have a detrimental effect on spore germination in the inoculum. The influence of the

substrates in which spores are frequently applied to seeds is unknown. If spores are applied in a slurry to the seed coat and allowed to dry, viability may be adversely affected and with epicotyl extension many spores may be carried away from the root vicinity on the testa. Where a spore bearing matrix or coating on the seed is used, this must detach from the seed on moistening, leaving the inoculum in close contact with the root system. The effects of seed viability, both before and after coating with inoculum sources, have not previously been studied. The influence of seed fresh weight on seedling development is well known, high seed weights promoting maximum growth, but its influence on potential development of mycorrhizas on seedlings has not been investigated.

These factors have been studied with the aim of producing granulated P. mugo seeds, using basidiospores of S. luteus as an inoculum source.

7.2 MATERIALS AND METHODS

The effects of spore concentration, spore 'carriers' and seed weight on mycorrhiza formation were studied using semi-aseptic synthesis techniques (as described in Chapter VI, Section 2) under growth room conditions (16 hour day, 20°C unless otherwise stated. They were set up as follows:

1 Spore concentration effects. The effects of both fresh and stored spores, applied to seeds in various concentrations, were investigated.

Pots were filled with previously sterilised soil, using 5 pots/treatment and 5 seeds/pot (later thinned to 3 seeds/pot). Aliquots of fresh spores, diluted in distilled water, were pipetted close to each seed (1 ml/seed). The dilutions were prepared to give the following spore concentrations/ml: 10^4, 10^5, 10^6, 10^7, 10^8 and seeds were also coated with a spore slurry. The latter had approximately 5 x 10^7 spores/seed (as determined by haemocytometer counts).

Stored spores were added in a sand/vermiculite 'carrier' (50 cc/pot) dug into the top 2 cm of soil in each pot. Quantities of spores used were as follows:

A Cool Stored Spores

1	0.01 g spores	per pot
2	0.10 g spores	
3	0.15 g spores	

B Freeze Dried Spores

1	0.01 g spores	per pot
2	0.05 g spores	
3	0.10 g spores	

C 0.01 g Cool Stored Spores
+ 0.05 g Inositol
+ 0.05 g Nicotinic Acid

D 0.01 g Cool Stored Spores
+ 0.05 g Inositol

E 0.01 g Cool Stored Spores
+ 0.05 g Nicotinic Acid

} per pot

F Control - 'carrier' only, no spores.

The chemical stimulants used in C, D and E were dissolved in 10 ml of sterile, de-ionised water and added to the 'carrier' medium just before application to the pots. Haemocytometer counts indicated that 0.1 g of spores contains approximately 4.0×10^8 spores.

2 Spore 'carrier' effects. Pots were set up as above (but using nine per treatment) and the following spore 'carriers' were added, each containing either 0.1 g or 0.01 g of spores.

A No 2 grade vermiculite.
B Washed river sand.
C Broken River soil.
D Distilled water.
E Control (no carrier, no spores)

All 'carriers' were steam sterilised (A, B and C on two consecutive days) and the appropriate quantity of spores added before transferring 50 cc of each to the pots and mixing the 'carrier' well with the top 2 cm of soil. This experiment was set up in a glasshouse (average temperature 16°C, normal day length) and samples from three pots/treatment were assessed for mycorrhiza formation at two, three and four month intervals.

3 Seed weight effects. P. mugo seeds were classed into four groups;

A $\geqslant$ 0.01 g

B 0.007 - 0.01 g

C 0.005 - 0.007 g

D $\leqslant$ 0.005 g

In each group 30 seeds were weighed and the group mean weight determined. Five pots per treatment were set up (each containing 5 seeds) and to three of these 0.01 g of spores were added in 50 cc of sand/vermiculite 'carrier'. The remaining two control pots received only the 'carrier'. Cotyledon and root dry weights, total length of the primary root and number of mycorrhizal short roots/seedling were determined.

4 Seed viability. This study was carried out both in the laboratory with seeds being placed on sterile filter pads in petri dishes (incubated at 25°C) and in trays of washed sand in the glasshouse. Fifty seeds per treatment were used. The following seed types (obtained from the Forest Service) and treatments were investigated:

A FRES 1976/299

B FRES 1975/233

C FRES 1974/177

D Thiram dusted seeds

E FRES 1976/299 surface sterilised with 1.5% Sodium hypochlorite or 10 volume Hydrogen peroxide. Seeds were shaken 10 minutes with the hypochlorite and one hour with the peroxide, then thoroughly washed in sterile distilled water.

Seed germination was assessed at regular intervals, seeds with radicles $\geqslant$2 mm long being considered as germinated. Granulated seeds (discussed in Section 7.3.5) were investigated in a similar manner.

5 Seed granulation. This study was carried out in conjunction with Fruitgrowers Chemical Co. Ltd. (Port Mapua, Nelson) who prepared the granulated seed samples. The seed sample used was *P. mugo*,

FRES 76/299 and these were inoculated as follows:

Reference No	
E516/1	Seed not inoculated, coated with finely ground, pH adjusted, peat equivalent to 50 percent of seed weight.
E516/2	Coated as for 1, inoculated (see inoculation rates below) with spores collected in 1976.
E516/3	Coated as for 1, inoculated with spores collected in 1977.
E516/4	Coated as for 1, inoculated with freeze dried spores collected in 1977.
E516/5	Coated as for 1, inoculated at low(*) rate with pulverised, freeze dried hymenial tissue.
E516/6	As for 5 but inoculated at high(†) rate.
E516/7	As for 5 but granulated with slow release N and P fertiliser at a rate equivalent to seed weight.

E516/8 As for 7 but inoculated at high rate.

E516/9 As for 7 but fertiliser equivalent to twice the seed weight.

E516/10 As for 9 but inoculated at high rate.

11 0.05 g freeze dried hymenial tissue applied in 50 cc sand/vermiculite 'carrier' per pot.

12 0.05 g cool stored spores applied in 50 cc sand/vermiculite 'carrier' per pot.

The inoculation rates were:

Spores (E516/2, 3, 4) 2 g spores (approximately 8.0×10^{11} spores) per 100 g seed.

Hymenial tissue - (†) High rate - 8 g tissue per 100 g seed.
- (*) Low rate - 4 g tissue (approximately 2.1×10^{12} spores) per 100 g seed.

The granule fertiliser mixture comprised the following materials:

Lime reverted superphosphate - 75%

Isobutylidendiurea (IBDU)	-	15%
Inert Binding agents	-	10%

Five seeds/pot were planted in five pots/treatment (containing previously sterilised Broken River soil) and were thinned to three seedlings/pot after one month. The experiment was terminated after six months under glasshouse conditions.

In all the semi-aseptic synthesis experiments seedlings were assessed individually for numbers of mycorrhizal short roots ($\leqslant$ 4 mm in length) and total number of short roots. From this mycorrhiza formation was expressed as a percentage of total short root numbers.

7.3 RESULTS AND DISCUSSION

7.3.1 Effect of Spore Concentration on Mycorrhiza Formation

Seedlings inoculated with fresh spores were assessed for mycorrhiza formation after six months and the results are given in Table 7.1.

It is evident from these results that as the spore concentration was increased, more seedlings became infected and there were significant increases in the number of mycorrhizal short roots formed per plant. Poor mycorrhiza

formation was obtained with slurry coated seeds, probably the result of many spores remaining on the testa.

TABLE 7.1 EFFECT OF FRESH SPORE CONCENTRATION ON MYCORRHIZA FORMATION

Spore Concentration/Seed			10^4	10^5	10^6	10^7	10^8
Percent Mycorrhizal Short Roots per Plant	Spore Aliquot	A	0	7.8	21.3	31.7	46.0
		B	0	47	47	80	87
	Slurry Coated Seeds	A	2.4				
		B	33				
	Control		0				

A % mycorrhizas on infected seedlings.

B % of assessed seedlings showing mycorrhizal infection.

Seedlings inoculated with stored spores were thinned and assessed for mycorrhizas after three months. At this stage, however, mycorrhizal formation was erratic and sparse in all treatments. When the experiment was terminated after six months, most seedlings were mycorrhizal and the results are given in Table 7.2. Greater spore concentrations again have the effect of increasing the number of seedlings showing infection and the number of mycorrhizal short roots on treated seedlings. Compared with the previous experiment (Table 7.1) levels of mycorrhiza formation have decreased using stored spores, but these

were mixed in a large quantity of 'carrier' instead of being pipetted immediately adjacent to seeds, therefore effectively lowering the number of spores/seed.

TABLE 7.2 EFFECT OF STORED SPORE CONCENTRATION ON MYCORRHIZA FORMATION

			Spore Quantity (g) and Concentration			
			0.01 4.0×10^7	0.05 2.0×10^8	0.10 4.0×10^8	0.15 2.0×10^9
Percent Mycorrhizal Short Roots per Plant	1 Freeze Dried Spores	A	16.3 Hh	19.3 Ff	21.8 Dd	
		B	75	80	90	
	2 Cool Stored Spores	A	17.6 Gg		24.0 Bb	27.4 Aa
		B	85		85	90
	2 + Inositol + Nicotinic Acid	A	19.8 Ff			
		B	90			
	2 + Inositol	A	21.0 Ee			
		B	87			
	2 + Nicotinic Acid	A	22.9 Cc			
		B	87			
	Control		0			

A % mycorrhizas on infected seedlings.

B % of assessed seedlings showing mycorrhizal infection

Std Error = $\pm$ 0.3

Mycorrhiza formation was reduced by about 50 percent but 'carrier' volume was increased about 100 fold, therefore the addition of spores in a 'carrier' is more effective.

The freeze dried spores were less effective at forming mycorrhizas than cool stored spores and a similar result is seen in Table 6.3. Addition of inositol and nicotinic acid increased mycorrhiza formation, but proportionately greater increases were gained by increasing the spore concentration.

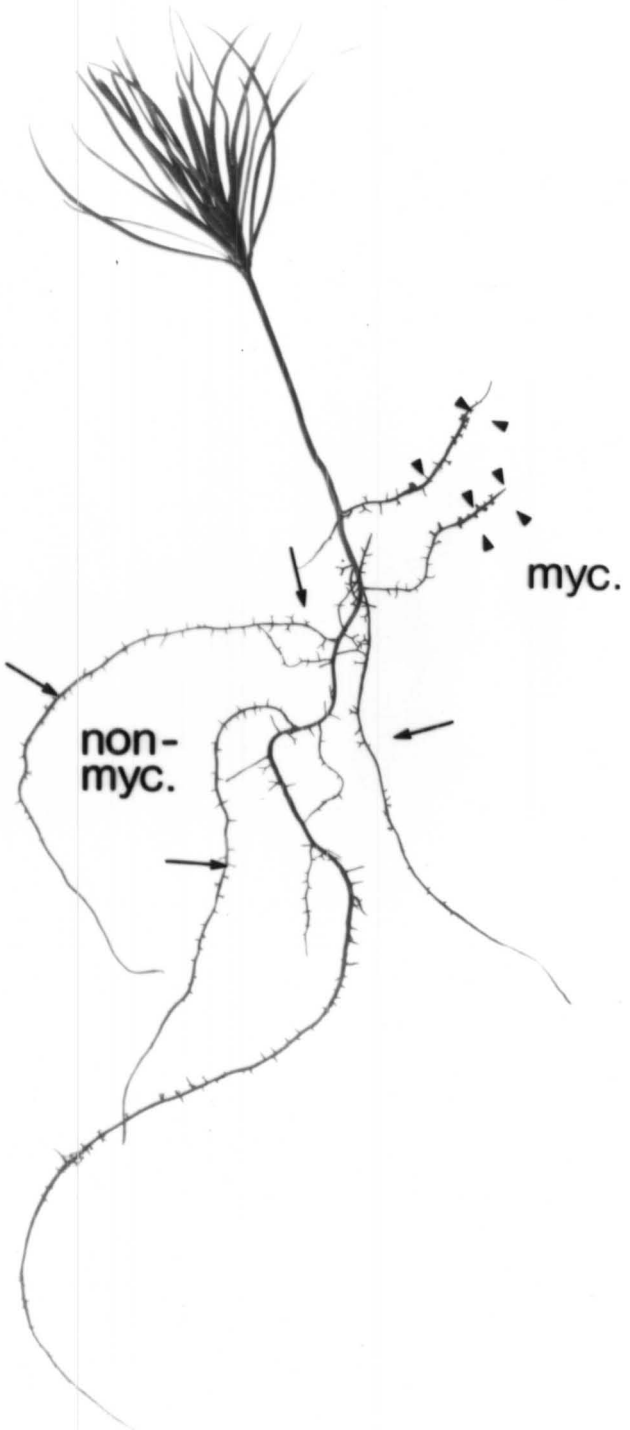
During assessment of the seedlings it was noted that many exhibited dichotomous roots which were apparently not mycorrhizal. It is thus difficult to determine the percentage of mycorrhizal short roots without exercising considerable care. This feature is evident in Fig. 7.1 where differences between mycorrhizal and 'non-mycorrhizal' short roots are not immediately obvious except where there is a definite web of hyphae on the root surface. In an attempt to determine the status of the 'non-mycorrhizal' short roots, dichotomies of both types were excised from several seedlings and surface sterilised. After washing with sterile water 10 mycorrhizal and 20 'non-mycorrhizal' dichotomies were transferred to plates of M40 agar and assessed after one week of incubation at 25°C for fungal growth. Seven mycorrhizal dichotomies had produced pure cultures of S. luteus after this period but no 'non-mycorrhizal' dichotomies exhibited any growth.

The production of 'non-mycorrhizal' dichotomies would, therefore, appear to be due to factors inherent in the seedling. They are found on both seedlings with and without mycorrhizas. On non-infected seedlings their presence is varied, some having few dichotomies and others

FIGURE 7.1 Mycorrhizal and non-mycorrhizal dichotomies.

These are often found on the same root system and care must be exercised to avoid including non-infected dichotomies (tailed arrows) when counting infected dichotomies (arrow heads). The latter often have a hyphal web over them and are hypertrophied and translucent in appearance. Natural size.

7-1



having many of these distinctive short roots. A typical highly mycorrhizal seedling is shown in Fig. 7.2, on which the true mycorrhizal dichotomies are made obvious by their hypertrophied form.

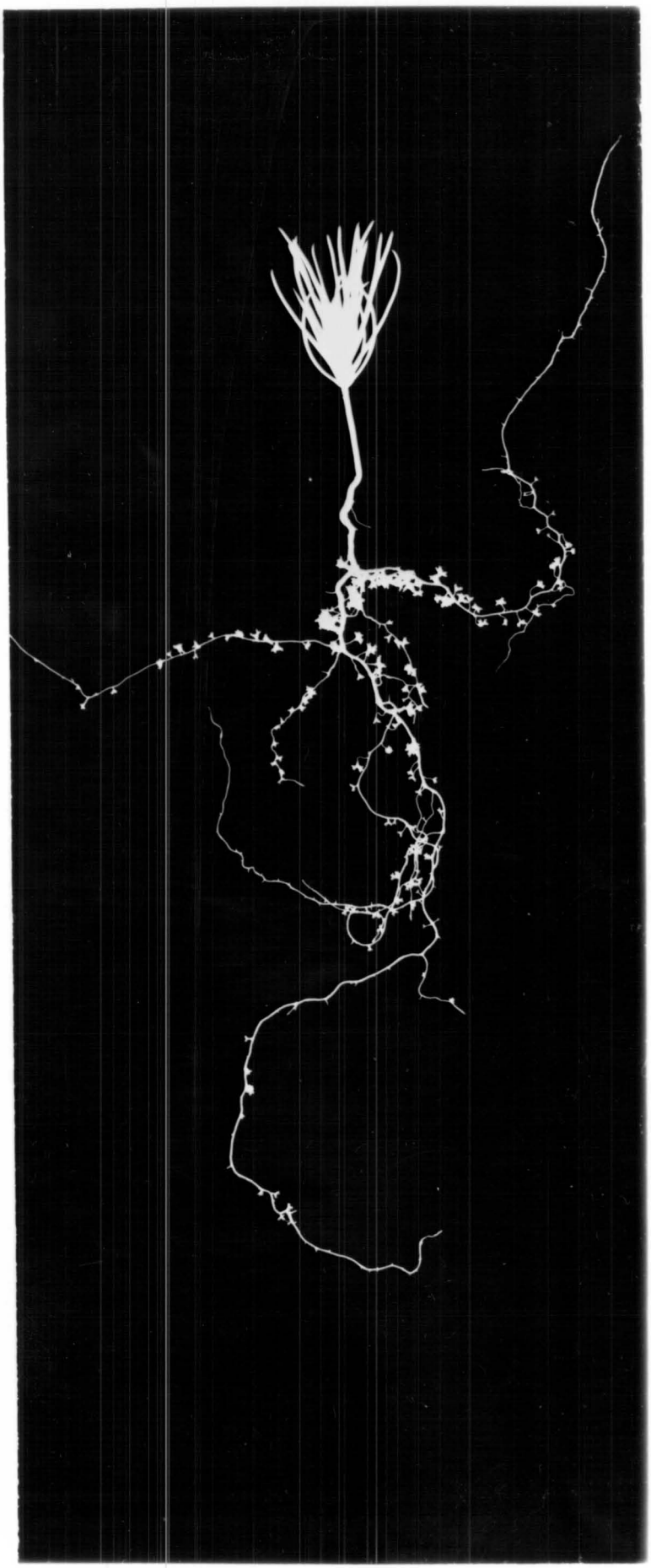
7.3.2 Effect of Spore 'Carriers' on Mycorrhiza Formation

The inoculum source for this study consisted of stored spores. At both the 0.1 g and 0.01 g spore application rates it was found that sand and vermiculite were the best 'carriers' (Graph 7.1). Soil and distilled water gave lower mycorrhizal percentages and this was particularly evident at the 0.01 g spore level where it is seen that treatments C and D showed no evidence of infection until the eighth week. With the spore inoculum a characteristic lag period before any visual sign of mycorrhizal infection was noted after inoculation. This lag was 6-10 weeks in duration with S. luteus spores and is probably due to spore dormancy and/or the time taken for host invasion by the fungus.

Marx (1976) concluded that a dry basidiospore inoculum of P. tinctorius mixed with a 'carrier' such as vermiculite, kaolin or soil was more effective than a suspension in water and suggested the possibility of water soluble germination inhibitors being involved. Marx also detected a similar lag period in infection after the seeds had germinated. A feature of mycorrhiza formation is an increase in this lag period with time of spore storage - in

FIGURE 7.2 Typical mycorrhizal seedling showing heavy infection of short roots. Note numerous multiple branched short roots near base of secondary roots where infection was initiated. Toward the tips of the secondary roots fewer multiple branched short roots are found but dichotomous roots became more common. Natural size.

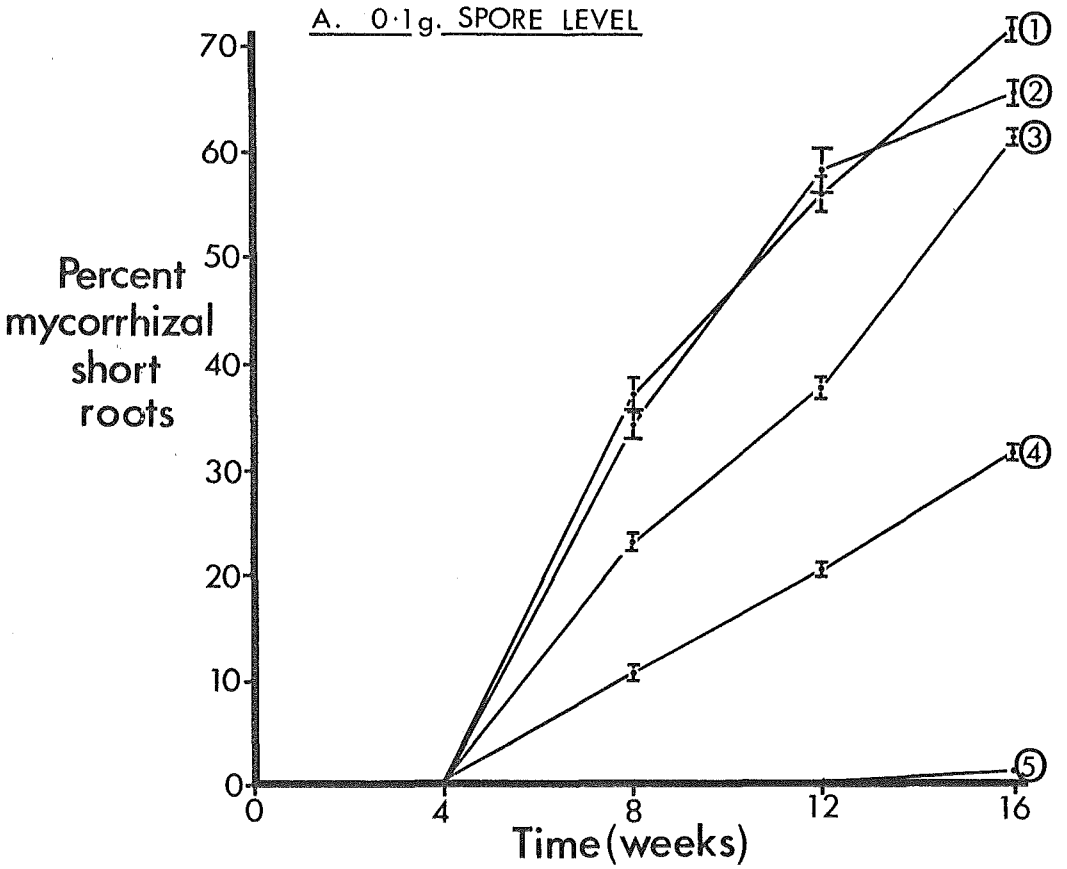
7-2

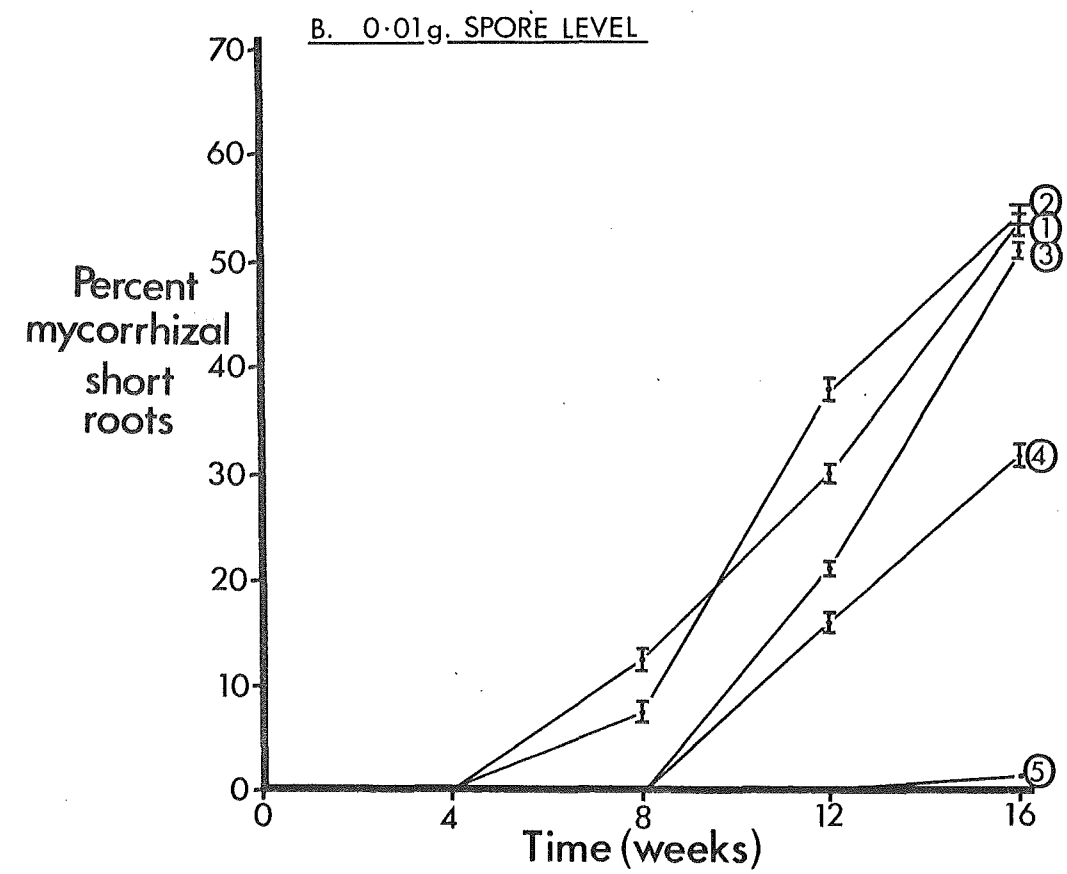


GRAPH 7-1 Spore 'carriers' are as follows:

1 Sand
2 Vermiculite
3 Soil
4 Distilled Water
5 Control

GRAPH 7-1
Effect of spore 'carriers' on mycorrhiza formation.





some studies seedlings never developed mycorrhizas although other seedlings in the same pots were infected. This was particularly noticeable where old stored spores were used.

7.3.3 Effect of Seed Weight on Mycorrhiza Formation

The mean weights of seeds in each of the four classes described in Section 7.2 were calculated and are shown in Table 7.3.

TABLE 7.3 SEED CLASS MEAN WEIGHTS

	Seed Class			
	⩾0.01 g (1)	0.007-0.01 g (2)	0.005-0.007 g (3)	⩽0.005 g (4)
Mean Seed Weight (g)	0.014	0.009	0.006	0.004

Stored spores were used to inoculate the seeds and seedlings were thinned after two months, those lifted out being checked for presence of mycorrhizas. Little infection had occurred in treatments (3) and (4) but mycorrhizas were present on seedlings from the heavier seed classes (see Histogram 7.1) and greater numbers of seedlings

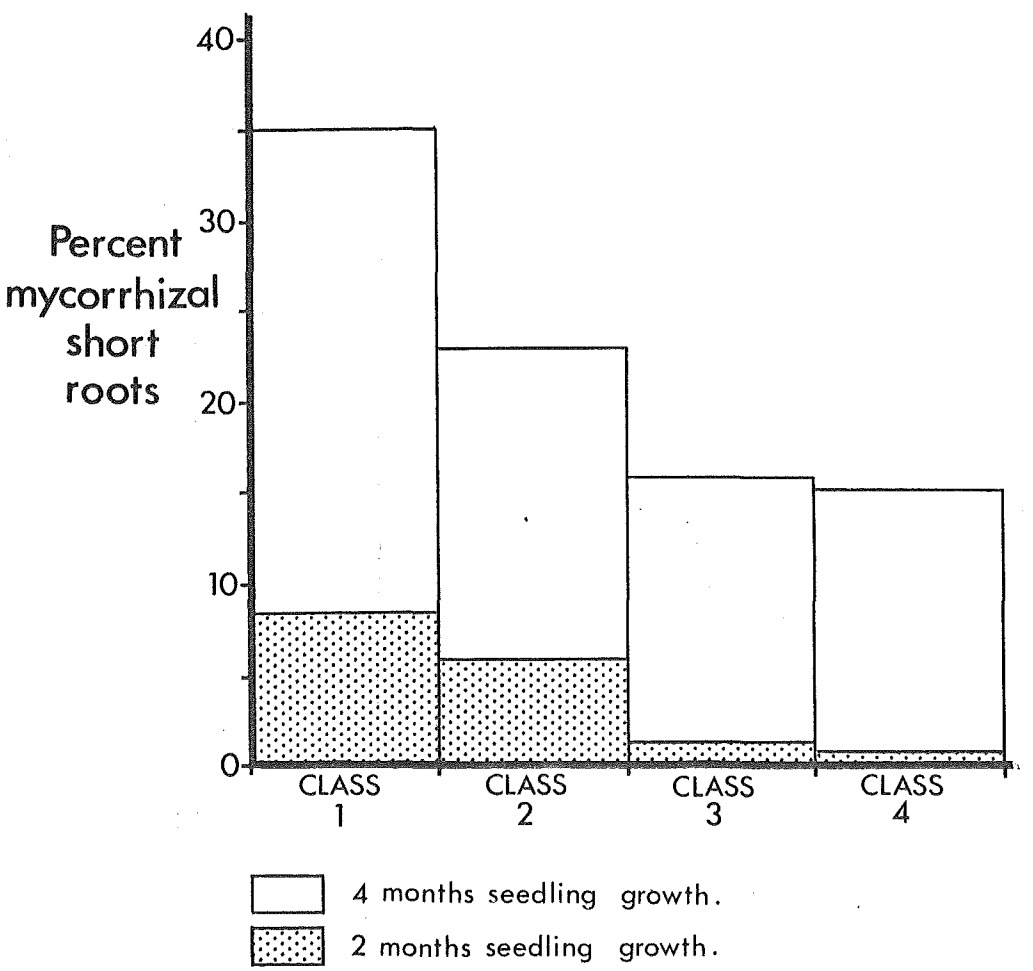
HISTOGRAM 7-1 Seed weight classes are as follows:

Class 1	$\geqslant$ 0.01 g
Class 2	0.007 - 0.01 g
Class 3	0.005 - 0.007 g
Class 4	$\leqslant$ 0.005 g

These are all measured as fresh weight.

Std Error = $\pm$ .05 percent.

HISTOGRAM 7-1
Effect of seed weight on mycorrhiza formation.



mycorrhizal. After four months all seedlings were mycorrhizal but again those from the heavier seed classes had more infected roots. The other parameters measured are shown in Table 7.4.

TABLE 7.4 EFFECT OF SEED WEIGHT ON SEEDLING ROOT LENGTH AND ROOT/SHOOT DRY WEIGHT

		Seed Class				Mean of Treatments 1-4	
		(1)	(2)	(3)	(4)		
Primary Root Length (mm)	Mycorrhizal	15.0	12.3	11.6	11.7	12.7	± 0.3
	Control	15.2	14.0	12.3	11.7	13.3	
Shoot Dry Weight (g)	Mycorrhizal	0.052	0.038	0.025	0.019	0.033	± 0.004
	Control	0.050	0.033	0.024	0.017	0.031	
Root Dry Weight (g)	Mycorrhizal	0.031	0.019	0.011	0.010	0.018	± 0.002
	Control	0.032	0.020	0.016	0.013	0.020	

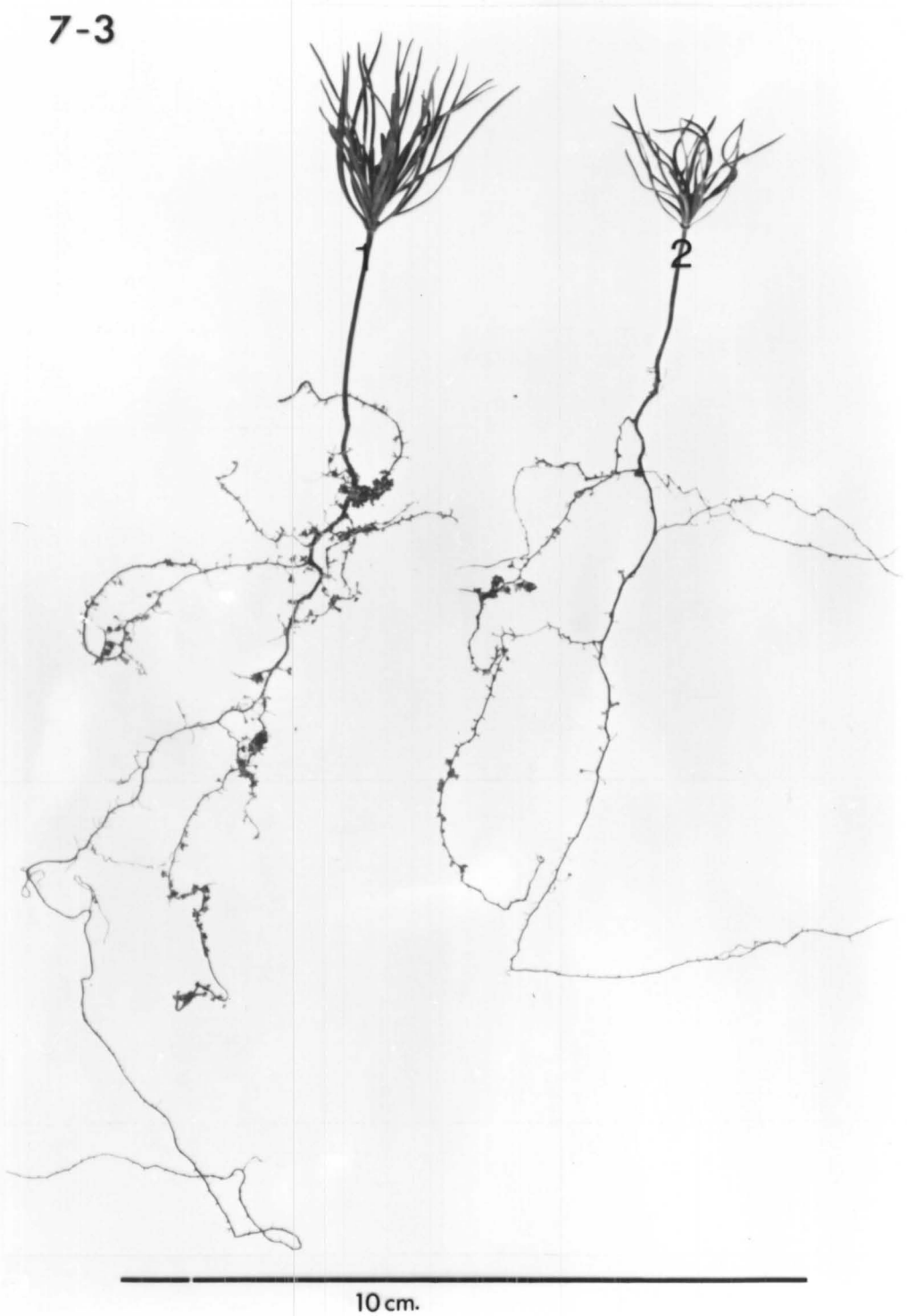
Significant decreases were detected in root length and seedling dry weight as seed weight decreased (see Fig. 7.3) for both control and mycorrhizal seedlings. No significant differences were noted between mycorrhizal and control seedlings, as seen in the above table, probably because of the slow growth of P. mugo. Longer periods of growth may increase differences between mycorrhizal and 'non-mycorrhizal' seedlings but would also

FIGURE 7.3 - Effect of seed weight on mycorrhizal development and seedling growth.

Seedling 1 is typical of those developing from a Class 1 seed ($\geqslant$0.01 g weight). Many mycorrhizas are present and dense foliage, as a result of strong growth, is evident.

Seedling 2 is typical of those developing from a Class 3 seed (0.005 - 0.007 g weight). Few mycorrhizas have formed and the seedling is stunted with slightly chlorotic foliage.

Natural size.


7-3
1
2
10 cm.

allow more chance of accidental infection from airborne spores.

7.3.4 P. mugo Seed Viability

During the preceding studies seed viability was brought into question as germination was often erratic. The seeds used were tested for germination under laboratory conditions and the results are given in Table 7.5.

TABLE 7.5 P. mugo SEED VIABILITY (LABORATORY CONDITIONS)

	Seed Type					
	76/299	76/299 Surface Sterilised with H_2O_2	76/299 Surface Sterilised with Hypochlorite	75/233	74/177	Thiram Coated
Percent Germination	84	84	14	64	70	4

The effect of surface sterilants was also investigated as Trappe (1961) found that 30 volume H_2O_2 stimulated pine seed germination. No such effect was noted with the level of peroxide used (10 volume) but the hypochlorite had an adverse effect on germination, even with thorough seed washing after treatment. Many seeds treated

with hypochlorite had emergent radicles but development of these abruptly stopped and none were longer than about 2 mm. Maximum germination (84%) was noted in the 1976 seeds, those from 1975 and 1974 showing reduced germination potential. Thiram, coated on seeds to prevent 'damping off' of seedlings, markedly reduced germination and the same effect was seen in seeds tested for germination under glasshouse conditions (Table 7.6).

TABLE 7.6 *P. mugo* SEED VIABILITY (GLASSHOUSE CONDITIONS

	Seed Type				
	76/299	76/299 Surface Sterilised with H_2O_2	75/233	74/177	Thiram Coated
Percent Germination	74	76	60	64	18

A 5-10 percent reduction in germination was observed under glasshouse conditions and reduced germination was again evident in the older seed collections. The effect of thiram was less drastic, probably caused by leaching of the chemical from the sand in which the tests were conducted. Thiram still reduced germination

considerably, however, and its use (to aid seedling survival) is thus questionable.

7.3.5 *P. mugo* Seed Granulation Studies

Granulated seeds were assessed for mycorrhizal infection after six months growth but prior to this, seed viability studies (under the same conditions as reported in Section 7.3.4) were carried out to investigate whether germination was affected by the granulation process (Table 7.7).

TABLE 7.7 GRANULATED SEED VIABILITY (GLASSHOUSE CONDITIONS)

	Seed Type				
	E516/1	E516/3	E516/5	E516/7	E516/9
Percent Germination	62	60	54	46	10

A decrease of about 10 percent germination was observed after granulation (cf Table 7.6 - 76/299 seed type) and another 10-50 percent reduction occurred with the addition of the fertiliser mixture to the seed coat (E516/7, 9). Seeds treated with fertiliser appeared to

abort shortly after imbibition and radicle emergence - similar to the effect noted with hypochlorite. Some fertiliser treated seeds did germinate, but mycorrhiza formation on these was adversely affected. The result of granulation effect on mycorrhiza formation is shown in Table 7.8.

TABLE 7.8 EFFECT OF SEED GRANULATION ON MYCORRHIZA FORMATION

PART 1 - SPORE BASED INOCULUM

	Seed Treatment					
	E516/1	E516/2	E516/3	E516/4	11	12
A	0	4.3	4.0	3.6	3.2	11.2
B	0	42	50	25	33	100

PART 2 - FREEZE DRIED HYMENIUM INOCULUM

		E516/5	E516/7	E516/9	Mean of Treatments (Inoculum)
Low Rate of Application	A	2.7	3.4	0	3.05
	B	50	8	0	
		E516/6	E516/8	E516/10	
High Rate of Application	A	4.5	2.5	0	3.50
	B	42	25	0	
		Minus Fertiliser	Plus Fertiliser	2 x Fertiliser	
Mean of Treatments (Fertiliser) - A		3.6	2.9	0	

A % mycorrhizas on infected seedlings.

B % of assessed seedlings showing mycorrhizal infection.

Mycorrhizas were formed on seedlings from both the spore and hymenial inoculated seeds. The level of infection was low, but would be sufficient to act as a primary inoculum source for neighbouring roots of the developing seedling. Spores collected in 1976 and 1977 showed similar levels of mycorrhizal infection, but freeze dried spores infected fewer seedlings which also showed decreased numbers of mycorrhizal short roots. The high rate of hymenial inoculum application (containing approximately 4.2×10^{12} spores/100 g seed) gave infection levels equivalent to those of cool stored spores (with approximately 8.0×10^{11} spores/100 g seed). Doubling the hymenial inoculum application rate did not greatly increase the numbers of mycorrhizas formed on seedlings. The highest level of mycorrhizal infection recorded was from the cool stored spore 'carrier' inoculum, in which spores were mixed in with the soil that the seedlings grew in.

Further investigation of the granulated seeds included haemocytometer counts of spore numbers in the coating material. These were as follows (Table 7.9):

TABLE 7.9 SPORE NUMBERS IN GRANULATED SEEDS

	Seed Treatment					
	E516/3	E516/4	E516/5	E516/6	E516/7	E516/8
Mean No of Spores/ Seed	3.9×10^5	4.8×10^5	6.7×10^4	9.6×10^4	7.7×10^4	9.2×10^4

The high and low rate hymenial inoculum granulated seeds show spore counts differing by approximately 2.5×10^4 spores (E516/5, 7 cf E516/6, 8). Under microscope examination many of these spores appeared to be immature, probably accounting for the small difference between the treatments in mycorrhiza formation. The cool stored and freeze dried spore inoculated seeds had higher spore counts which would indicate that, in the granulated seeds, the hymenial inoculum is more efficient at producing mycorrhizas and that freeze dried spores are particularly ineffective in this role.

Spore viability was investigated to determine the effect of the granulation process on the inoculum source. Seed coatings were removed and stained with acridine orange as described in Chapter VI, Section 2. Numbers of spores with and without fluorescence were counted and compared (Table 7.10).

TABLE 7.10 SPORE VIABILITY IN GRANULATED SEEDS

	Cool Stored Spores	Granulated Seeds					
		E516/3	E516/4	E516/5	E516/6	E516/7	E516/8
Percent Viable Spores (non-fluorescing)	46.0	30.0	16.0	20.0	20.0	3.0	4.0

A typical micrograph of fluorescing cool stored spores can be seen in Fig. 7.4 and after incorporation in a seed coating in Fig. 7.5. Spores from the freeze dried hymenial inoculum were fewer in number but their fluorescence tended to be less intense (Fig. 7.6 A, B) and their colour appeared dull green/orange, in contrast to the bright yellow/orange to red colour of those from cool stored collections. This would suggest that spore survival rates may be higher in the hymenium inoculum in spite of decreased spore numbers.

7.3.6 Discussion

Mycorrhizal inoculum trials utilising fresh spores showed that infection was greatest at spore levels that would be considered quite high. At this level the young seedling has abundant mycorrhizal roots which would provide a continuing source of inoculum as the pine grows. The use of stored spores at equivalent levels was very effective considering that the spores were dispersed in a large bulk of 'carrier', probably because developing roots were continually in contact with the inoculum source. Where inocula are applied close to the seed, root infection can only occur early in root development and this type of mycorrhiza formation is often characterised by clusters of infected short roots around the bases of the oldest secondary roots. This is in contrast to more widespread mycorrhizal short root formation over all roots when the inoculum is supplied in a 'carrier'. Freeze drying of the inoculum was found to decrease mycorrhizal formation. Nicotinic acid and inositol stimulated mycorrhizal

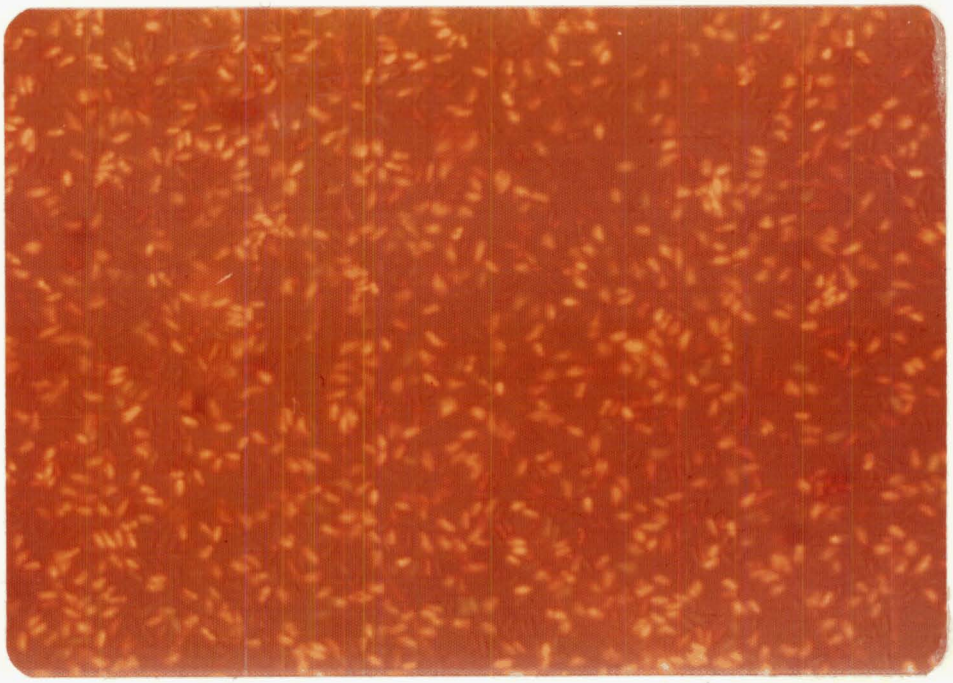
FIGURE 7.4 Fluorescing cool stored spores.

Both dead (orange) and alive (green) spores are visible. Acridine orange stain. x250

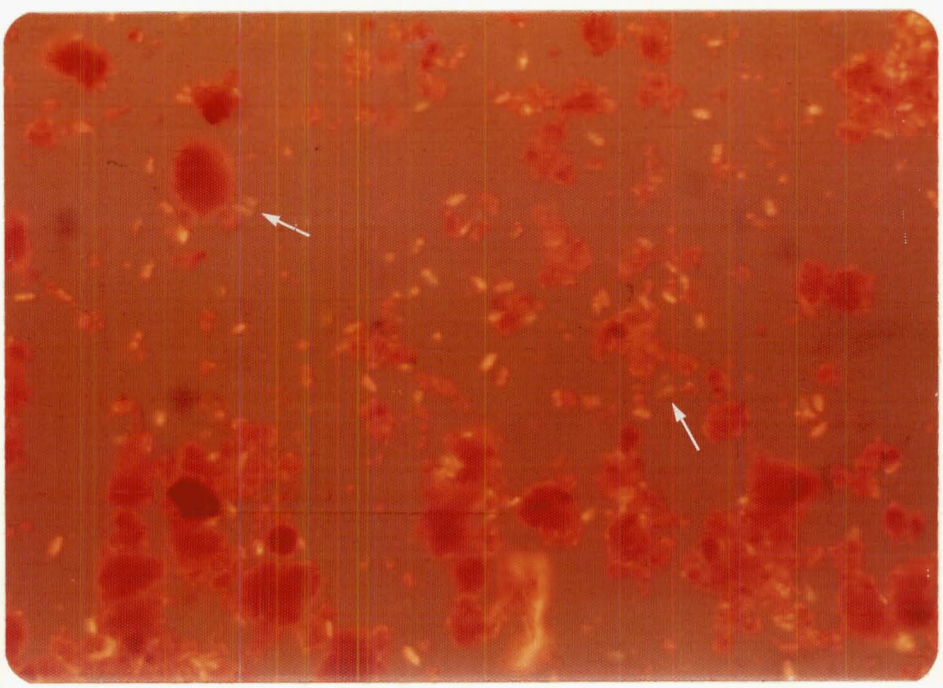
FIGURE 7.5 Cool stored spores incorporated in a granulated seed coating.

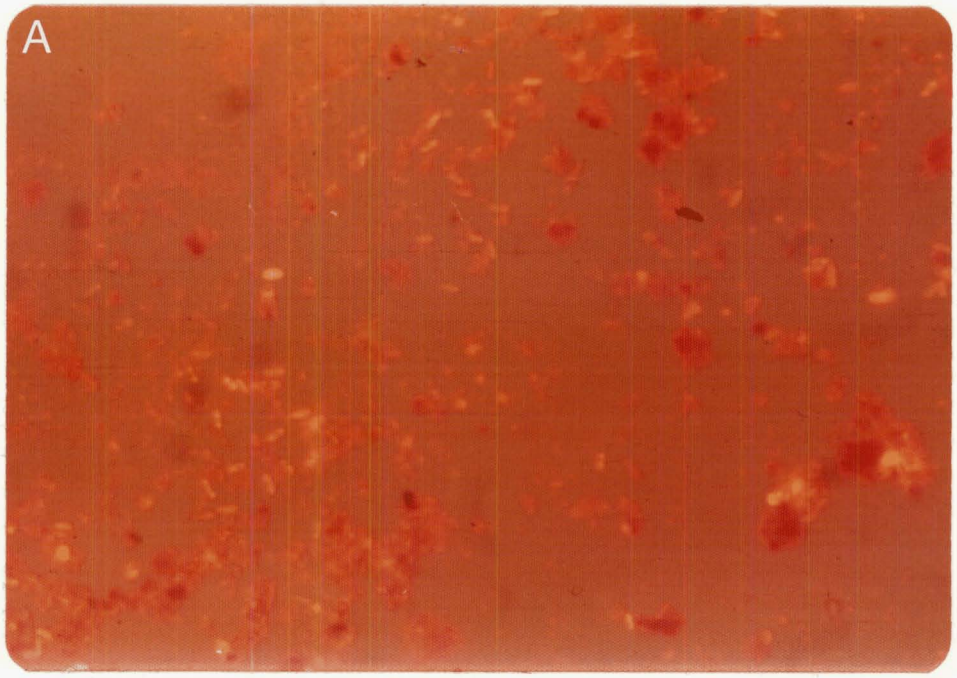
Most appear yellow or orange but some are still alive (arrowed). x250

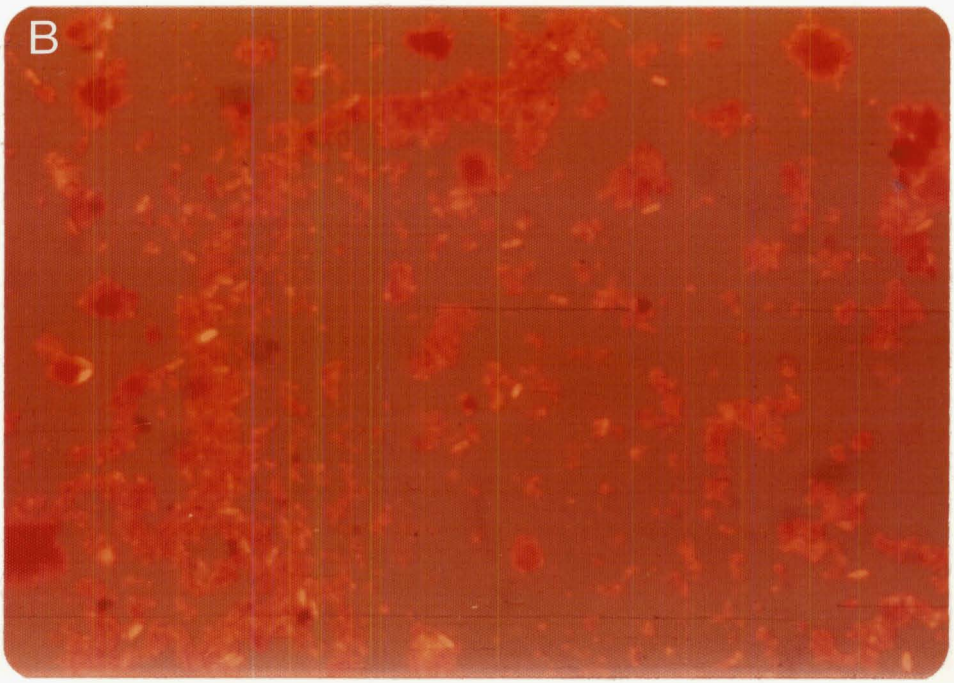
7-4



7-5




A


B

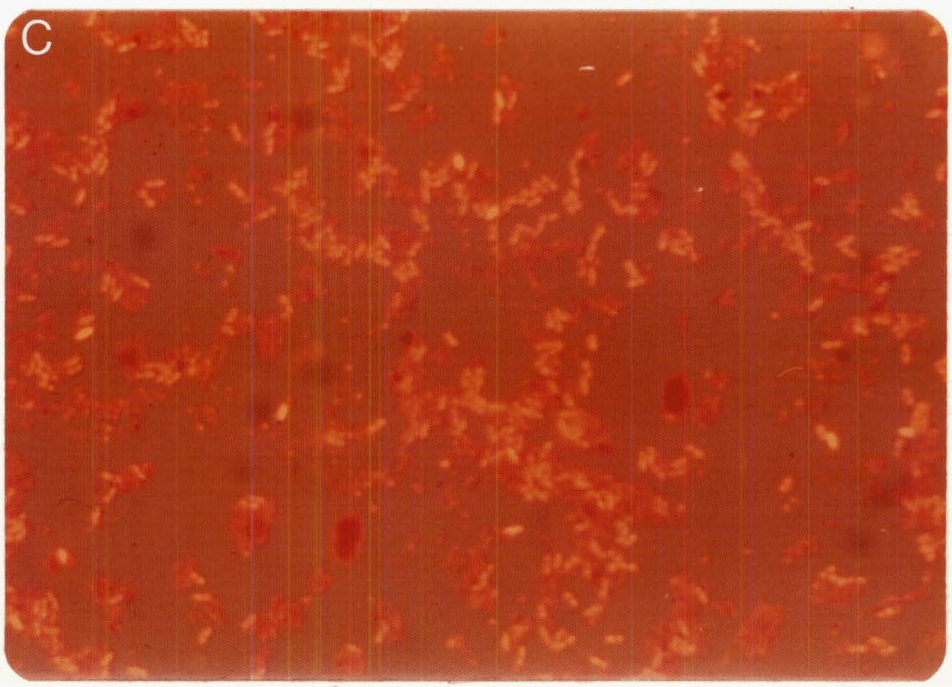

C

FIGURE 7.6 Freeze dried spores incorporated in a granulated seed coating.

A Freeze dried hymenial tissue (low rate of application).

B Freeze dried hymenial tissue (high rate of application).

C Freeze dried mature spores.

Note greater numbers of spores in C and their bright orange staining. Spores in A and B all show green bands in a central position. x250

production but to a lesser extent than was seen when the spore concentration was increased.

Various spore 'carriers' were investigated and it was found that sand and/or vermiculite appeared to provide the best conditions for seedling inoculation; soil or water 'carriers' giving lower infection rates. Lowered spore concentrations and extended storage inoculum times increased a lag period noted between seed germination and mycorrhiza formation. This lag must be reduced to a minimum in high country situations to allow maximum mycorrhiza formation and growth during the short growing season.

Seed weight has a marked effect on mycorrhizal formation in P. mugo. High weight seeds invariably showed greater numbers of mycorrhizal roots and significantly increased root sizes and root/shoot dry weights on seedlings developing from them. Seed viability is another important factor in seed oversowing that was investigated. Seeds inoculated by granulation of a spore bearing matrix on to the testa showed reduced viability thus it is essential to use a fresh seed source with a high germination level.

The use of thiram for the prevention of damping off reduces seed germination considerably, therefore its use may not be justified. Applications of fertiliser with the inoculum had detrimental effects both on seed germination and on mycorrhizal formation, similar to the effect of high soil fertility reported by Marx et al. (1977) on loblolly pine mycorrhizas. Lamb and Richards (1974) have, however, shown

that additions of fertiliser (as superphosphate) can increase mycorrhiza formation but that this was dependent on the initial soil nutrient status.

The hymenial inoculum, in spite of having lower spore counts, produced good mycorrhizal infection when applied at the high rate in granulated seeds, as compared to that produced by cool stored spore inocula. This was also evident when spores from these coatings were stained with acridine orange and observed since fewer bright orange/red spores (dead) were noted. The freeze dried hymenial inoculum is easy to prepare in bulk, although it appears to contain fewer mature spores, and is more easily handled than pure spore inocula. Freeze drying of this material seems to have less effect on the basidiospores it contains (these being still attached to the basidia) than on mature spores collected after their release from the sporophore.

The granulated seeds produced, therefore, show potential for revegetation of eroded slopes providing both spore and seed viability are maintained to ensure quick establishment of mycorrhizal seedlings.

CHAPTER VIII

SCANNING ELECTRON MICROSCOPE STUDY OF MYCORRHIZAL INFECTION

8.1 INTRODUCTION

Conifer mycorrhizal root systems have been studied by a number of workers, including a detailed light microscopic study of Pinus species mycorrhizas by Hatch and Doak (1933). Investigations on the diversity of mycorrhizal form in pines with different myco-symbionts were made by Laiho (1965), Mikola (1965) and Wilcox (1968). Root colonization was shown to vary from the ectomycorrhizal association to ectendo- and pseudomycorrhizal forms. T.E.M. studies have revealed the close association between host and myco-symbiont in the balanced mycorrhizal state (Hofsten, 1969; Strullu, 1976) but apart from the recent studies of yellow poplar endomycorrhizas by Kinden and Brown (1975, 1975a, 1976) few comprehensive investigations of mycorrhizas using the S.E.M. have been made. This study was concerned with colonization of the developing short root by the myco-symbiont and the internal morphology of mycorrhizal roots.

8.2 MATERIALS AND METHODS

Soil was obtained from a forested site 1,100 metres

above sea level in the Craigieburn Range at Broken River, in which mycorrhizal duff, with S. luteus, was present. Soil from a nearby unforested site, sterilised as described Chapter VI, Section 2, was also obtained. Seedlings (2-24 months old) of P. mugo were grown in this soil. Roots of seedlings from the duff infected soil, from sterile soil inoculated with basidiospores and from control, uninoculated soil were excised and prepared for scanning electron microscopy by the method of Kinden and Brown (1975). This method and some modifications made to it are outlined in Appendix 2. Specimens were critical point dried and mounted on aluminium S.E.M. stubs using double sided adhesive tape. They were gold coated in an Edwards 306 vacuum coating machine and examined with a Cambridge Stereoscan 600 S.E.M. operating at 15 or 25 Kv.

Roots prepared for T.E.M. observation were fixed in glutaraldehyde and osmium tetroxide as outlined in Chapter III, Section 2. After dehydration and araldite embedding they were sectioned, stained and examined with a Hitachi HS-75 T.E.M. (at 50 Kv). Other roots were excised and sectioned by freezing microtomy. These were kept in distilled water before rapid freezing in liquid nitrogen. Sections were cut and mounted in serial order on numbered slides before staining in lactophenol cotton blue and observation under a Leitz orthoplan microscope.

8.3 RESULTS AND DISCUSSION

8.3.1 Mycorrhizal Sheath and Hartig Net Formation

The infection process initiates at an early stage of development in the new lateral root. Fig. 8.1 shows a lateral emerging from the mother root with strands of fungal hyphae evident on its surface. Reference to Chapter V (Figs. 5.9,10,11) indicates that these hyphae can grow along the root surface forming a web over newly emergent short roots, or they may arise from germinated spores in the surrounding soil. As the new lateral grows, abundant hyphae are found on its surface adjacent to the mother root (Fig. 8.2). Higher magnification (Fig. 8.3) shows these are starting to form the mycorrhizal sheath with numerous interwoven hyphal strands. The tip of the short root still appears to be rapidly elongating and few hyphal elements are evident on it (Fig. 8.4) but the hyphae soon grow forming a dense web over the surface (Fig. 8.5). The sheath consists of hyphae with the typical papillated surface noted previously (Chapter IV, Fig. 4.3) and it is probable that the fungal influence on the host, early in the lateral's development, induces dichotomy of the short root and cessation of its growth.

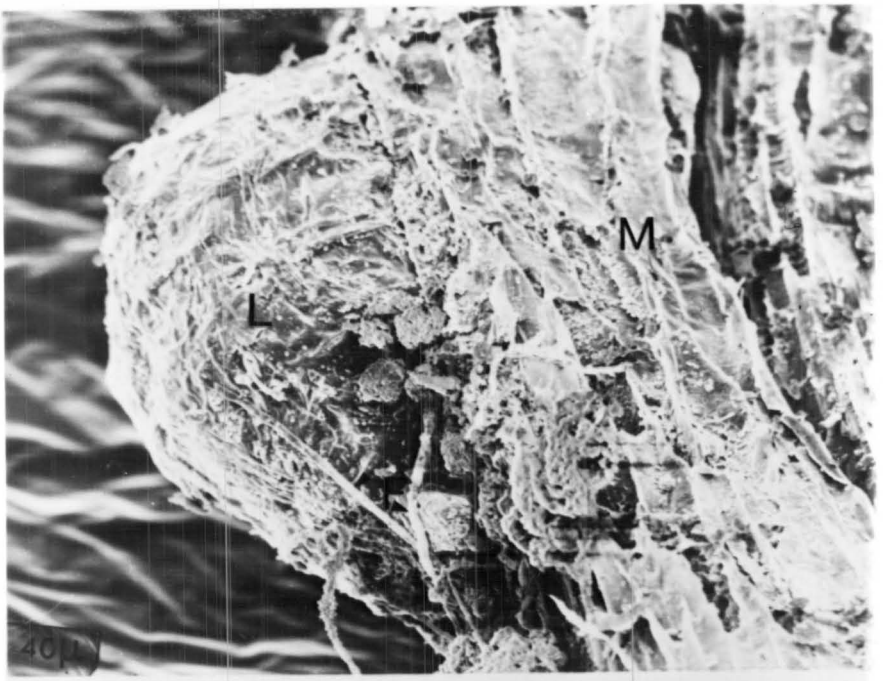
Figure 8.6 shows the surface of a short root with the sheath removed. Some hyphae remain and these appear to follow the line of cell junctions. The Hartig net is also evident. At high magnification the entry points of the fungus between host cells are seen (Fig. 8.7), again

FIGURE 8.1 Lateral (L) emerging from mother root (M). Note presence of fungal hyphae on root surface (F). x170

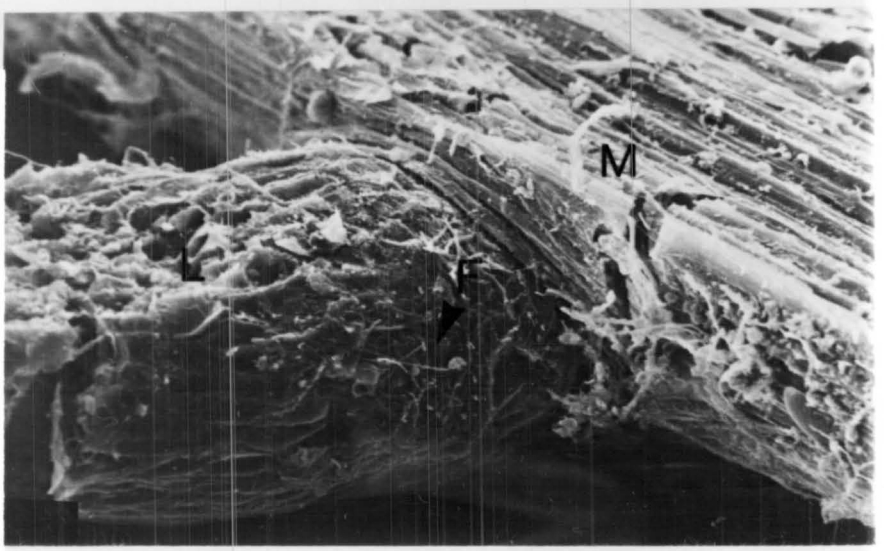
FIGURE 8.2 Older lateral (L) growing out of mother root (M). More hyphae are present on the root surface (F) and are beginning to form an interwoven network. x70

FIGURE 8.3 Close-up of junction between lateral (L) and mother root (M). Rudimentary sheath is evident (S) which may have originated by contact with hyphae on the mother root surface, or by infection from spores or Hartig net elements within the mother root. x170

8-1



8-2



8-3

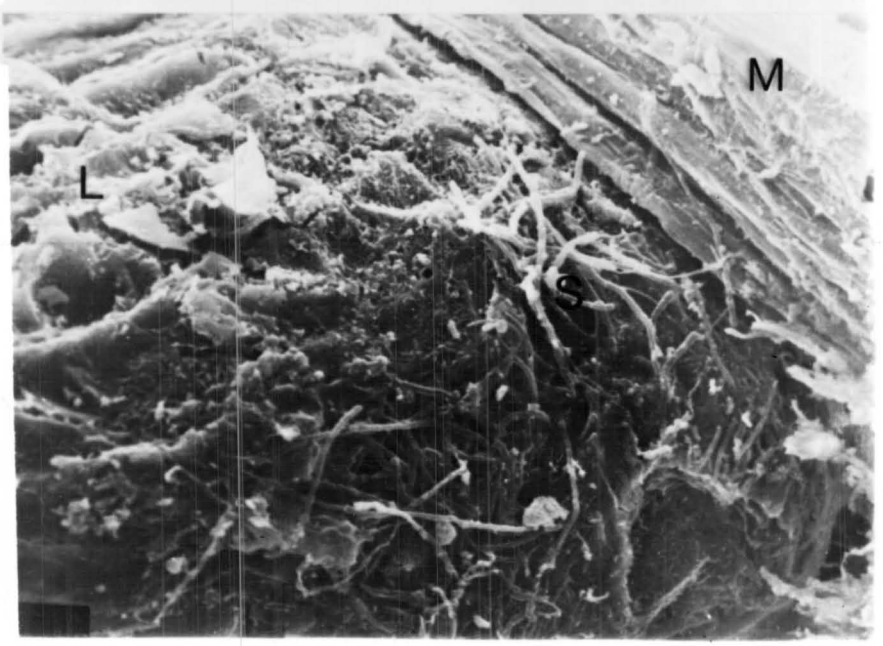


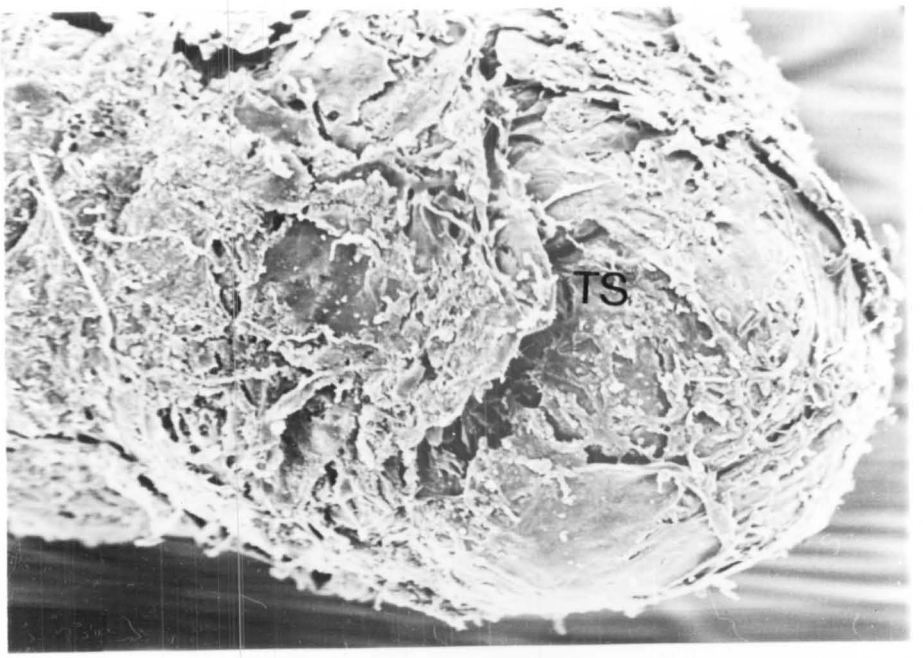
FIGURE 8.4 Short root tip (TS). Development of the sheath appears to be basipetal in nature as the tip is not well endowed with hyphae at this early stage. The short root has not ceased its initial rapid elongation, allowing completion of the sheath. x160

FIGURE 8.5

A Mycorrhizal short root with fully developed sheath. In this case the sheath (S) is loosely woven due to disturbance when cutting the sections, but in the top right (arrow) of the micrograph hyphae are closely adpressed to the root surface. Cortical cells (C) with Hartig net elements are also visible. x100

B Sheath hyphae at higher magnification (S). Papillae can be detected on the hyphal surfaces. x165

8-4



8-5

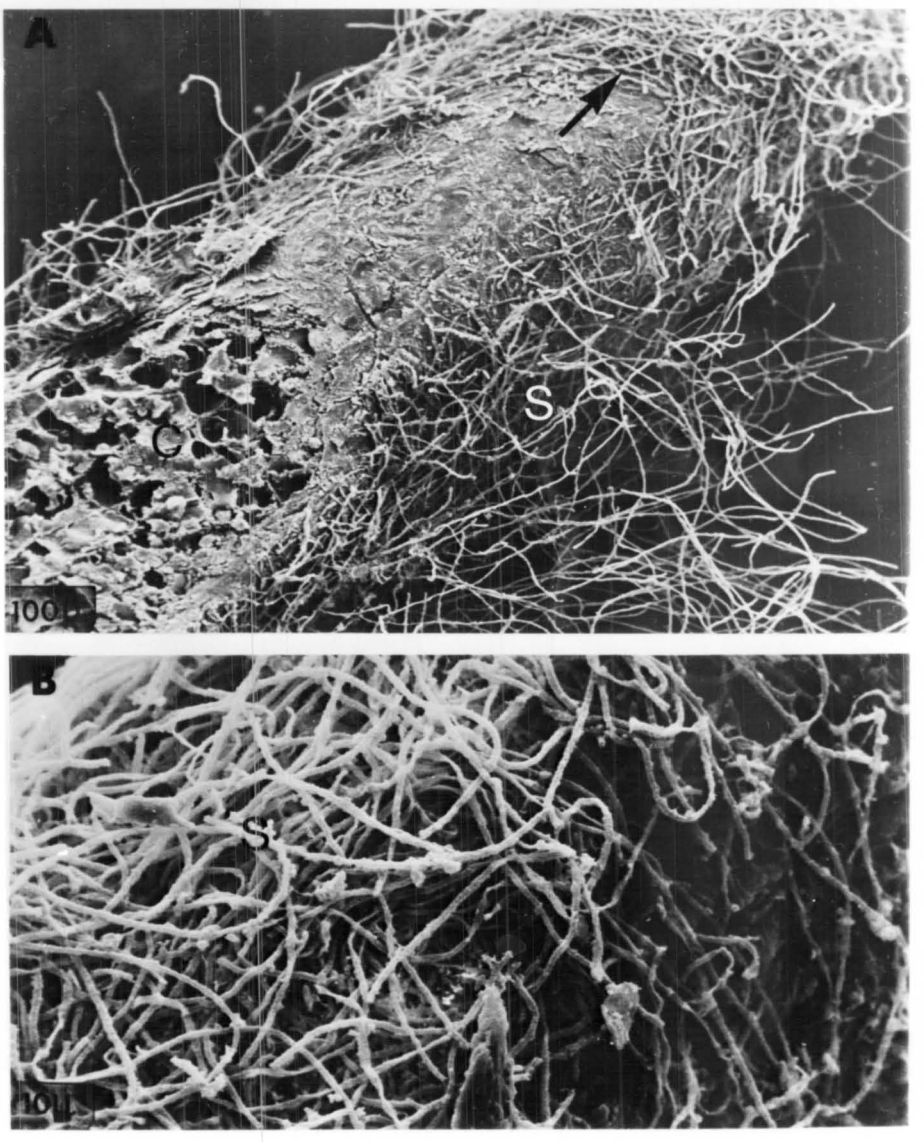
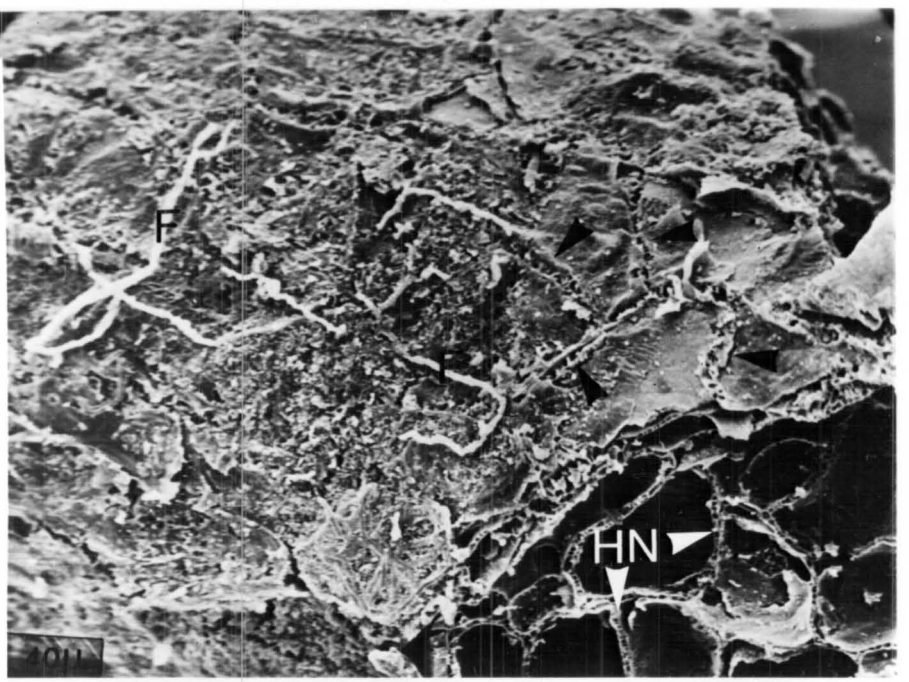


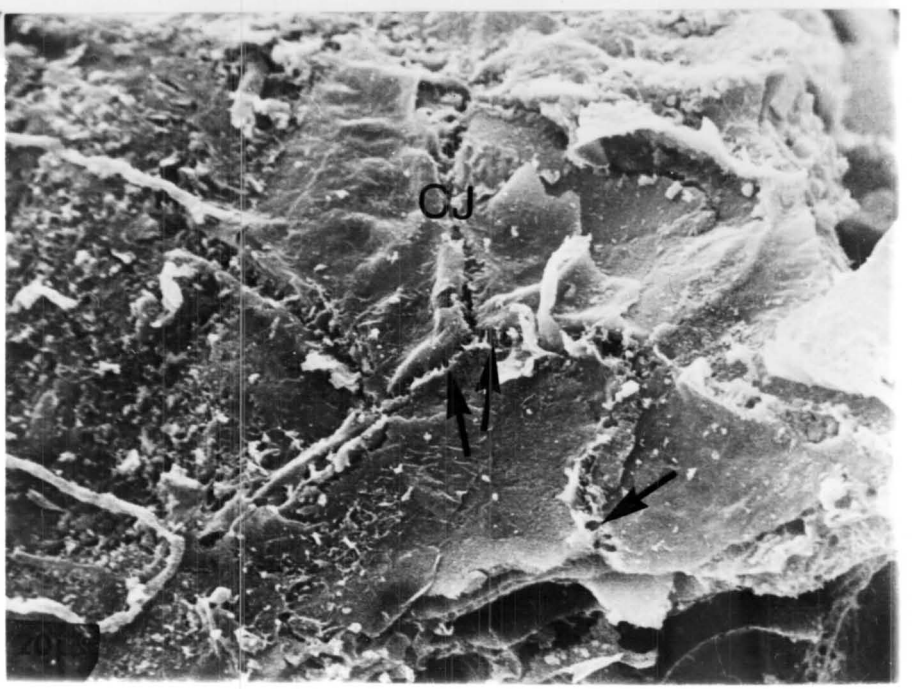
FIGURE 8.6 Short root surface from which sheath has been removed. Some hyphae are still present (F) and the Hartig net within the root is evident (HN). The junctions of underlying cells can be seen on the root surface (arrows). x360

FIGURE 8.7 Cell junctions (CJ). It is evident that these are the entry points of the mycorrhizal fungus into the root (arrows). x720

8-6



8-7



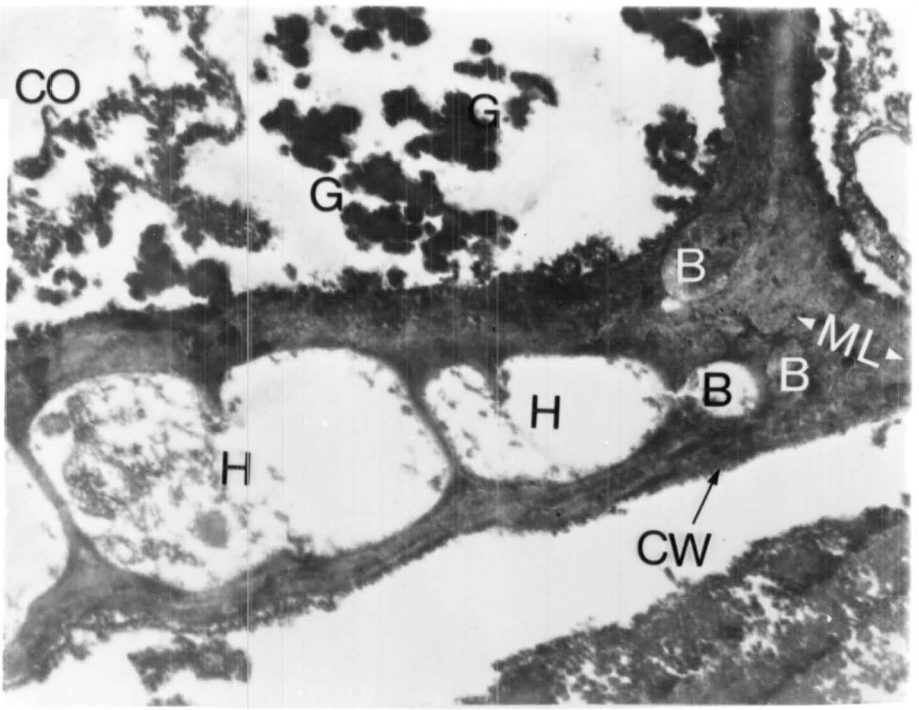
following the underlying cell walls between adjacent cells. In ectomycorrhizas the surface cell walls therefore remain intact but those between cells of the epidermis/cortex are penetrated by the symbiont. Cleavage of the cell wall occurs along the middle lamella as new hyphal buds are formed and preceding ones expand (Fig. 8.8). A feature of some mycorrhizas are bacterial cells found in close association with fungal hyphae on the root surface (Fig. 8.9).

In many roots the sheath is a loosely woven network of hyphae but mycorrhizas with a 'spiked' appearance are also seen (Fig. 8.10). The association of these spikes with the sheath is apparent in Figs. 8.11,12. At this stage of development these mycorrhizas could be classified as Ia (pseudoparenchymatous mantle cream-yellow to brown in colour with spikes, these being pointed, stiff and aseptate or with single septa) or Ha (soft mantle hyphae composed of loose strands, colourless or white-grey, around a pseudoparenchymatous layer, often with spikes) using Dominik's (1969) key. However, as mentioned by Trappe (1967) this system is based on broad morphological descriptions and as the mycorrhiza develops, it often passes through several sub-groups as presented by Dominik, thus care must be taken when making conclusions based on such a system. In transverse section the spikes are seen to arise from the outer sheath layers (Fig. 8.12) and in some cases are very dense (Fig. 8.13). An extensively spiked dichotomous short root is shown in Fig. 8.14. These hyphal protrusions may act as a substitute for root hairs, increasing the surface area of the symbiont considerably.

FIGURE 8.8 Hartig net development. In thin section the cell wall (CW) is seen to be cleaved along the middle lamella (ML) as hyphal buds (B) are formed and preceding hyphal elements (H) expand. The dark, granular deposits (G) in the adjacent cortical cell (CO) are thought to be tannins. x4000

FIGURE 8.9 Bacterial cells (B) found in close association with fungal hyphae (F) on a root surface. x400

8-8



8-9

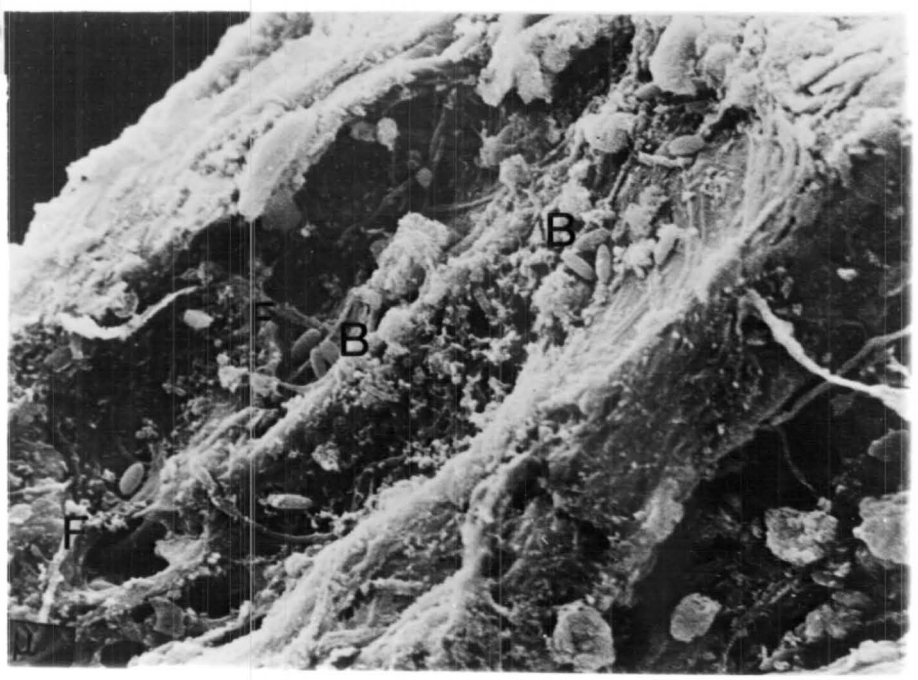
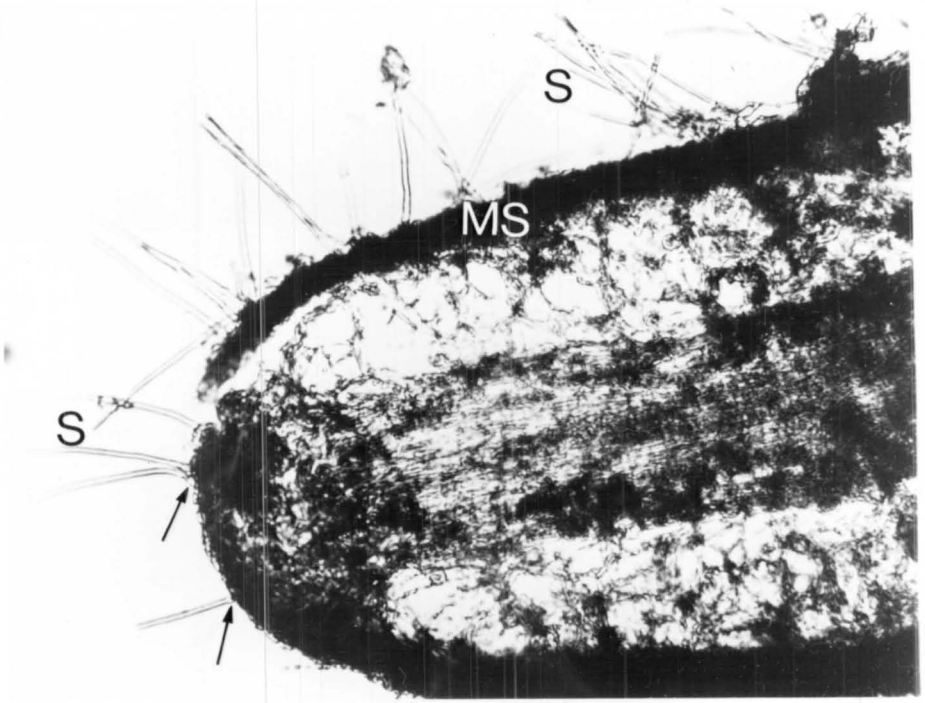


FIGURE 8.10 'Spiked' mycorrhizas (light micrograph). Hyphal spikes (S) can be seen originating from the mycorrhizal sheath (MS). The base of the spikes have a slightly bulbous appearance (arrows). x160

FIGURE 8.11 'Spiked' mycorrhizas (scanning electron micrograph). The same features as in 8.10 are shown. x165

8-10



8-11

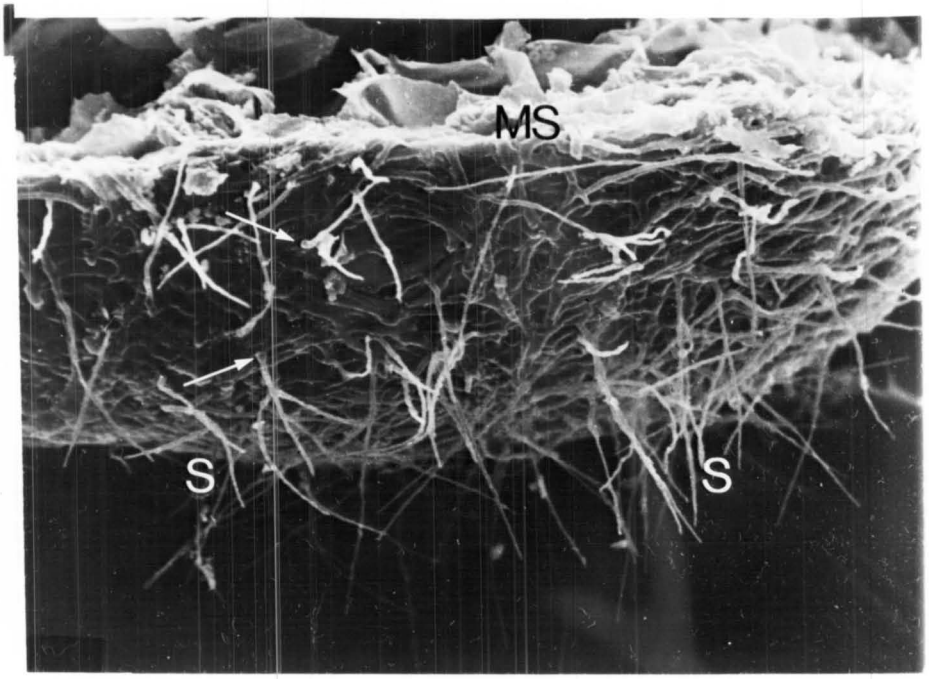
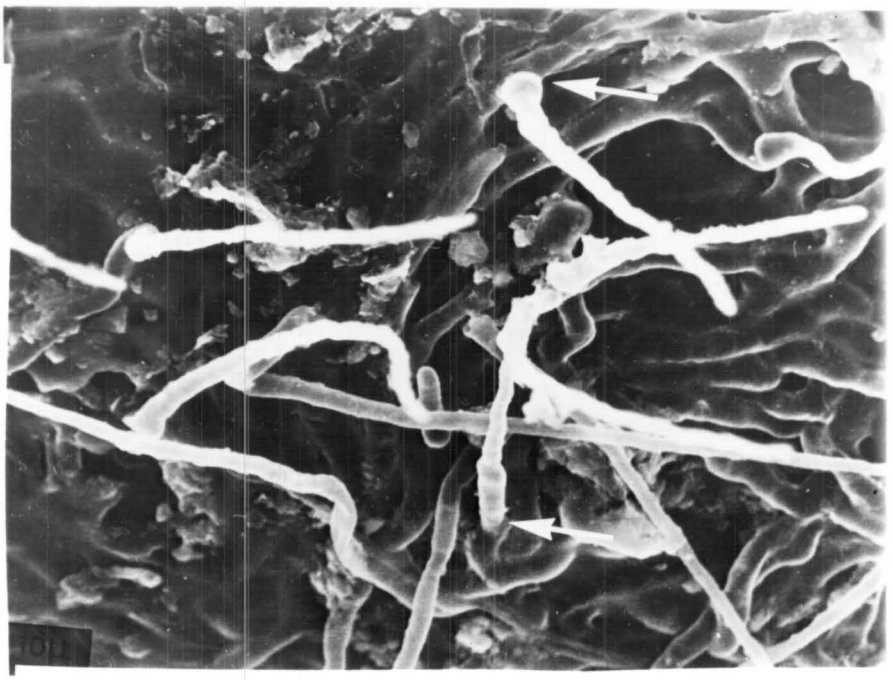


FIGURE 8.12 Sheath 'spikes' at high magnification. Note bulbous base (arrows) and attachment to sheath hyphae. x640

FIGURE 8.13 Sheath 'spikes'. Note density of the spikes in this root and their close association with the sheath (MS) - see inset. x170
Inset: x700

8-12



8-13

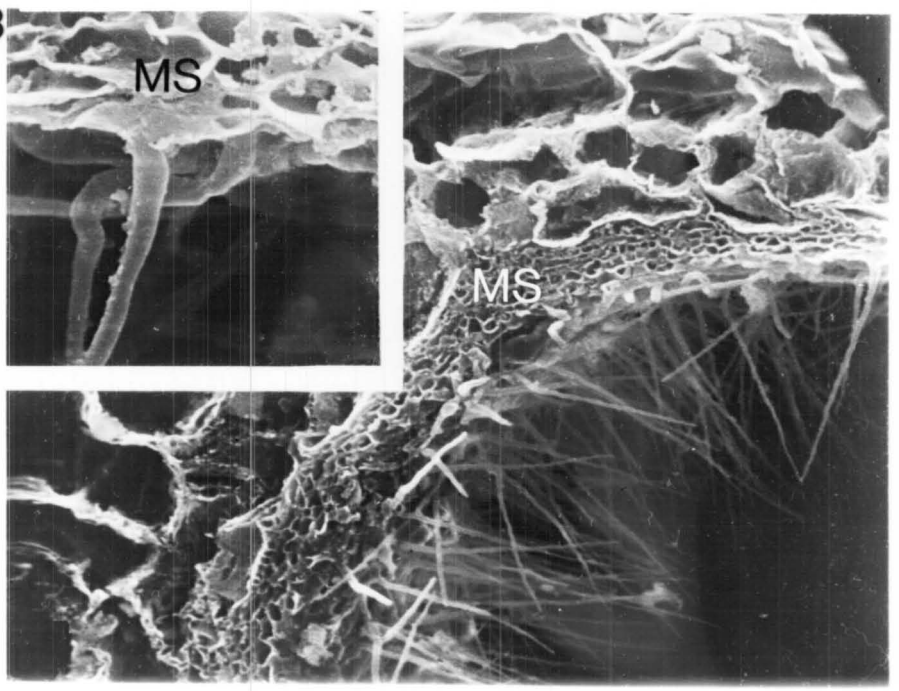
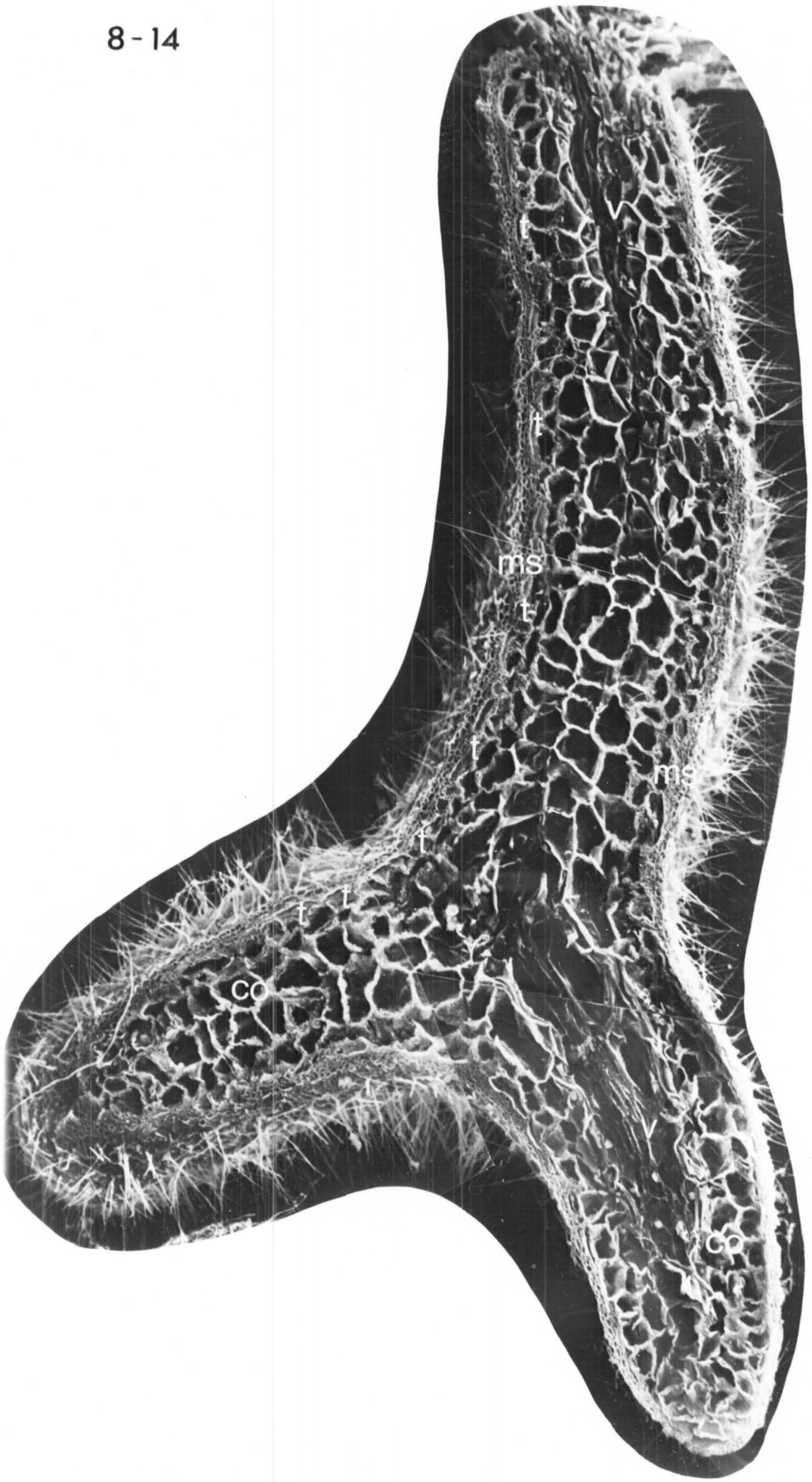


FIGURE 8.14 Extensively spiked dichotomous short root.

These hyphal protrusions may act as a substitute for root hairs, absent on mycorrhizal roots.

A well developed sheath (MS) is evident and the tannin cell layer (T) can be seen inside this. Between the cortical cells (CO) Hartig net elements are present and in a central position the vascular tissue (V) is seen. x60

8 - 14



The infection process was followed with longitudinal sections during root emergence. At the very early pre-emergence stage shown in Fig. 8.15 the developing lateral passes through root tissue containing visible Hartig net elements. These may be another source of infection for the new lateral. After the root emerges the Hartig net soon becomes well developed although the sheath is still not complete (Fig 8.16). The tip of the root has few hyphae covering it as it is rapidly elongating. Granular bodies, of unknown composition are often noticed in the mycorrhizal roots, particularly near the apical meristem and along the vascular bundle (Fig. 8.17). Mycorrhizal roots, whether non-dichotomous (Fig. 8.18) or dichotomous (Fig. 8.19), have no root cap and the root tip has a rounded appearance. At the extreme root tip only a narrow region of outer cortex is invaded by hyphae, but from 0.25 - 0.5 mm behind the tip, Hartig net elements can be seen to penetrate to the endodermis. In contrast, non-mycorrhizal roots have a distinct root cap and no fungal sheath or intercellular net is evident (Fig. 8.20). In all cases the mycorrhizal short root tip becomes extremely swollen as compared to the root base (Fig. 8.21). This is particularly evident where long roots have changed to mycorrhizal form (Slankis, 1967).

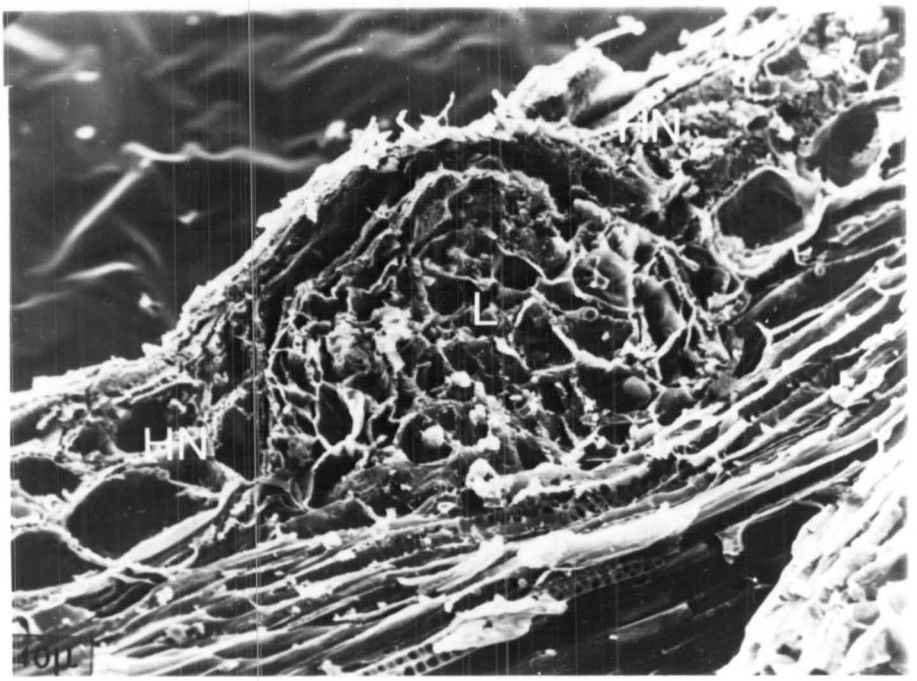
8.3.2 Development of the Hartig Net and Tannin Cells

A mycorrhizal root with a well developed sheath (many hyphae having clamp connections) and a Hartig net which has invaded the outer cortical cells is shown in Fig. 8.22. As the net extends into the cortex its older

FIGURE 8.15 Lateral root emergence (longitudinal section). At this pre-emergence stage the lateral (L) is passing through infected cortical tissue of the mother root (HN), this probably being a source of infection for the new short root. x90

FIGURE 8.16 Lateral root emergence. Shortly after emergence a rudimentary sheath (MS) is evident on the lateral's surface. x180

8-15



8-16

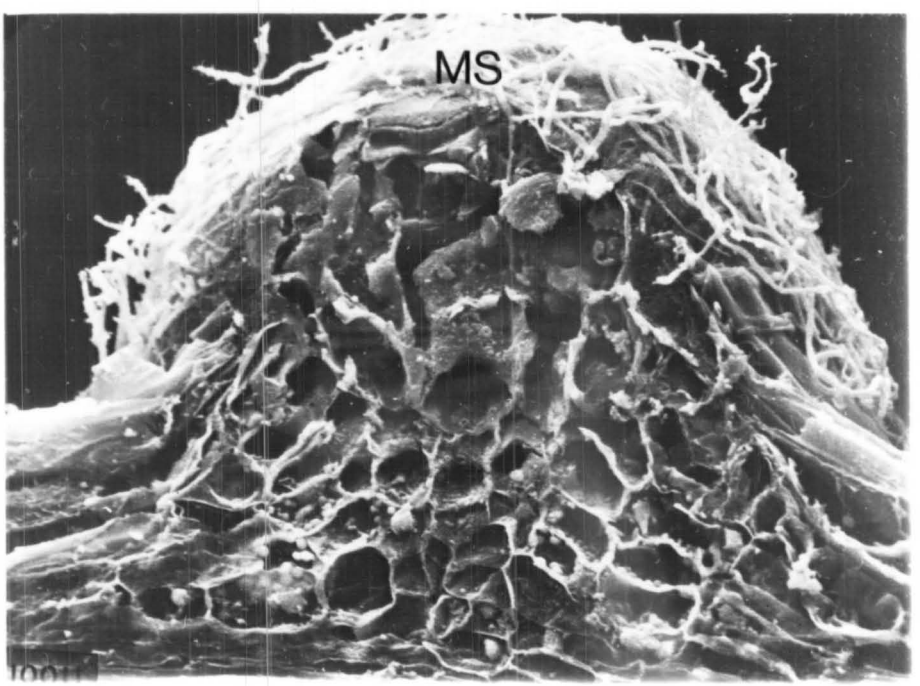
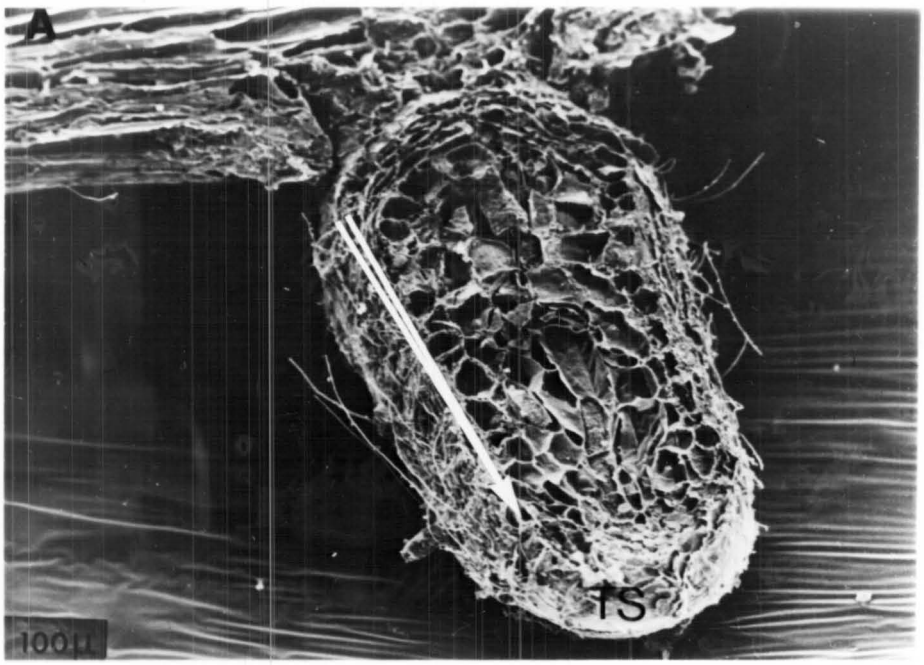


FIGURE 8.17 Developing lateral.

A Sheath and Hartig net are well developed toward the base of the lateral (arrow) but the tip has few hyphae covering it (TS). x80

B Near the tip of the lateral large granular bodies (G) are seen in the area of the root tip normally associated with meristematic divisions. x150

8-17



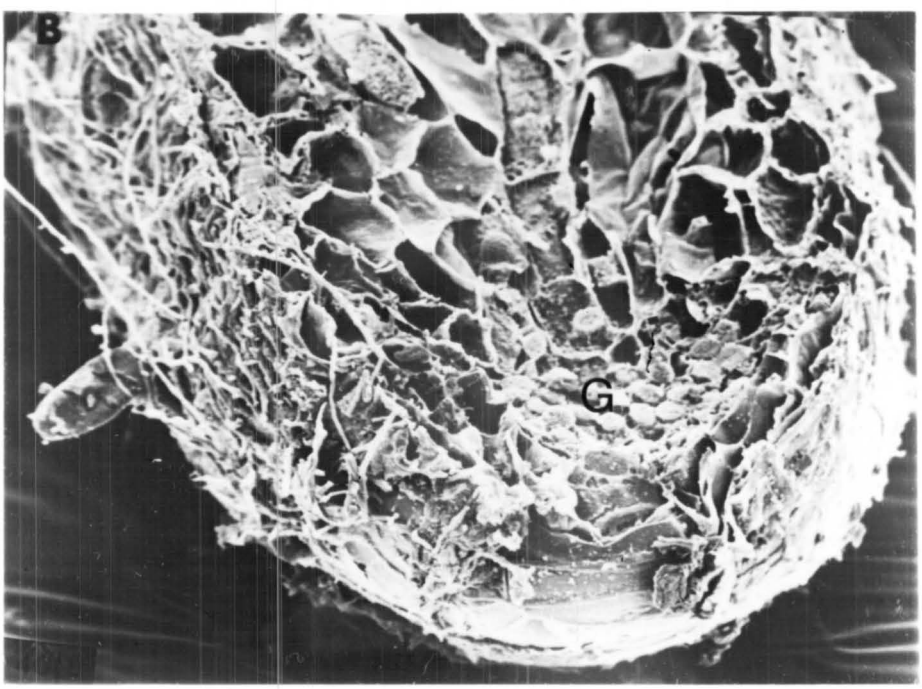


FIGURE 8.18 Non-dichotomous mycorrhizal root.

No root cap is present, this being replaced by the sheath (MS). Vascular tissue (V) reaches almost to the root tip where very little hyphal invasion occurs. From 0.25-0.5 mm behind the tip Hartig net elements can be seen to penetrate to the endodermis (dotted line).
x80

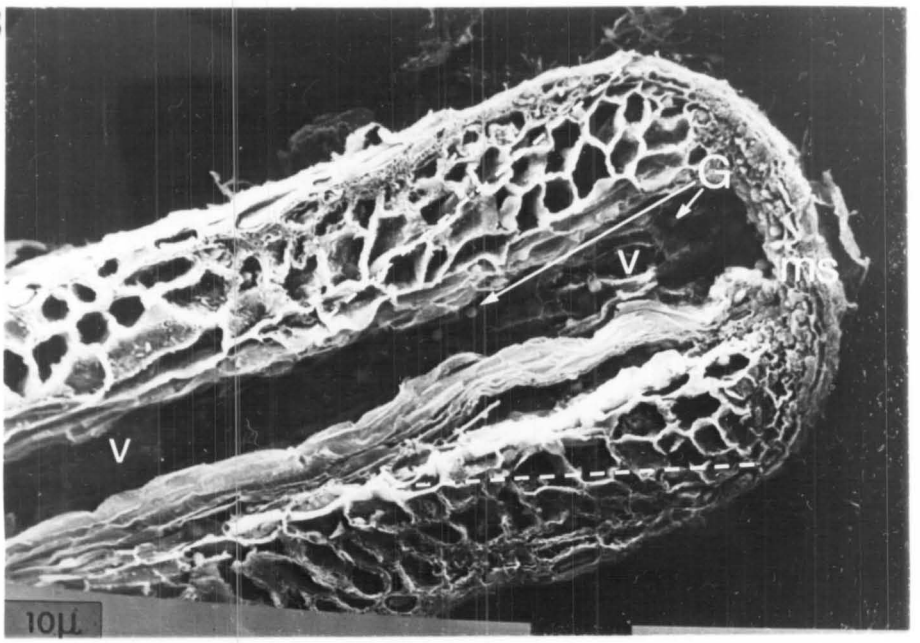
FIGURE 8.19 Dichotomous mycorrhizal root.

This shows similar features to the above. Note that in both roots granular bodies (G) are evident near the normal meristematic region, as in Fig. 8.17, and these are occasionally scattered back along the vascular tissue (V).
x50

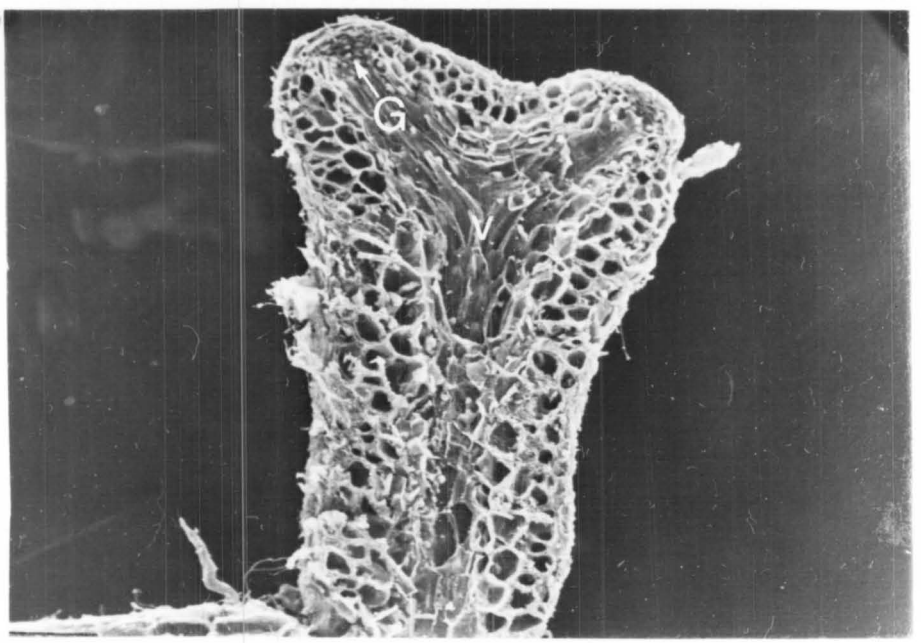
FIGURE 8.20 Non-mycorrhizal root.

Root cap (RC) is retained for protection of the meristematic region(M) during root growth.
x65

8-18



8-19



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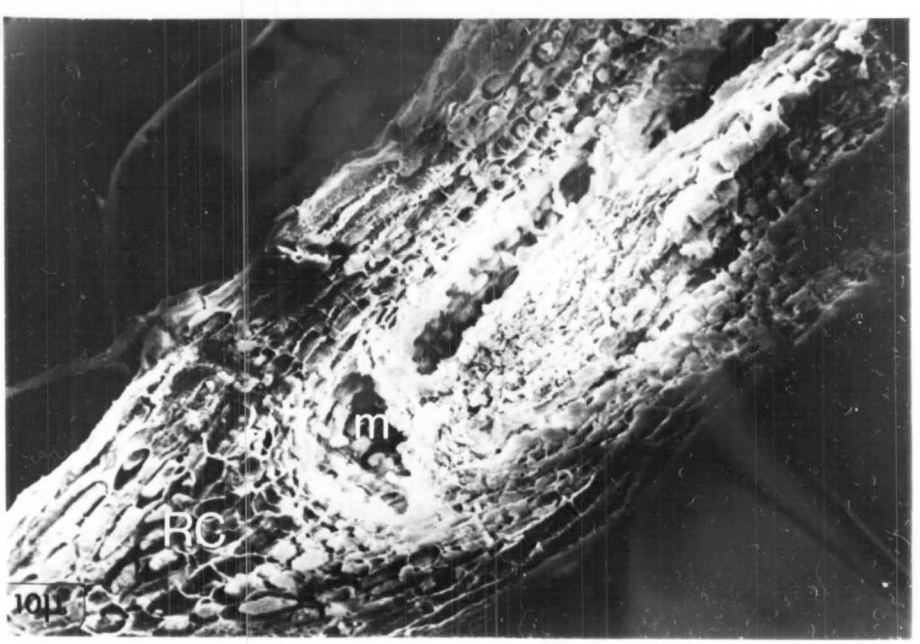
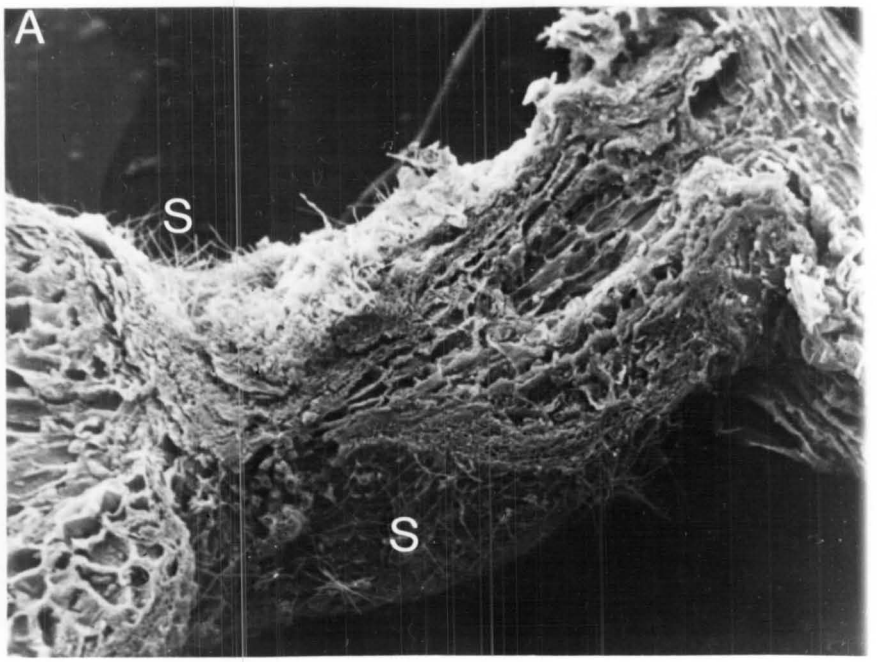


FIGURE 8.21 Swelling of mycorrhizal short root tip.

A Base of short root showing hyphal spikes (S) on surface. This area of the root is mycorrhizal but not swollen. x50

B Tip of same root. Sheath (MS) is distinct and cortical tissues of this part of the root (C) have become hypertrophied giving the swollen appearance characteristic of many mycorrhizas. x45

8-21



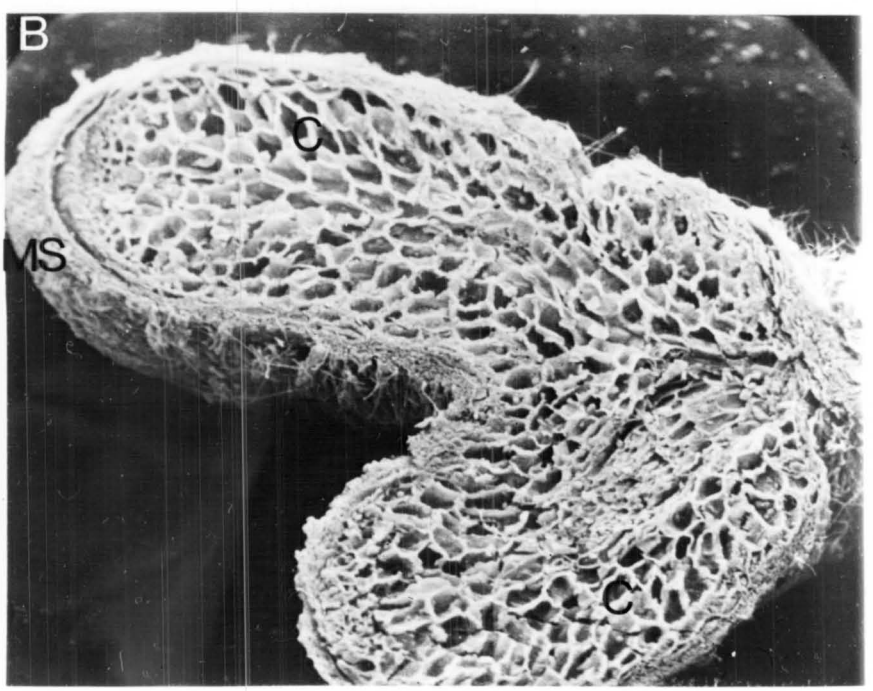


FIGURE 8.22 Hartig net development.

The sheath (MS), with papillated hyphae, is well developed and the Hartig net (HN) has invaded the cell walls between outer cortical cells (C). x180

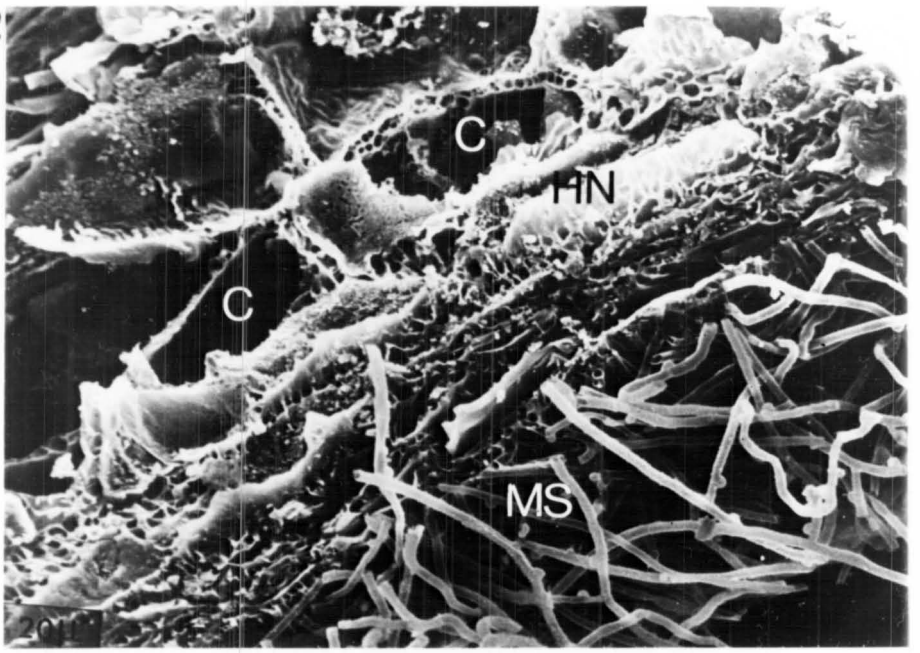
FIGURE 8.23 Hartig net extension.

A The sheath (MS) is again evident and Hartig net elements can be seen between the cortical cells (C). In the outer cortex these are two layered but further in they become single layered. x350

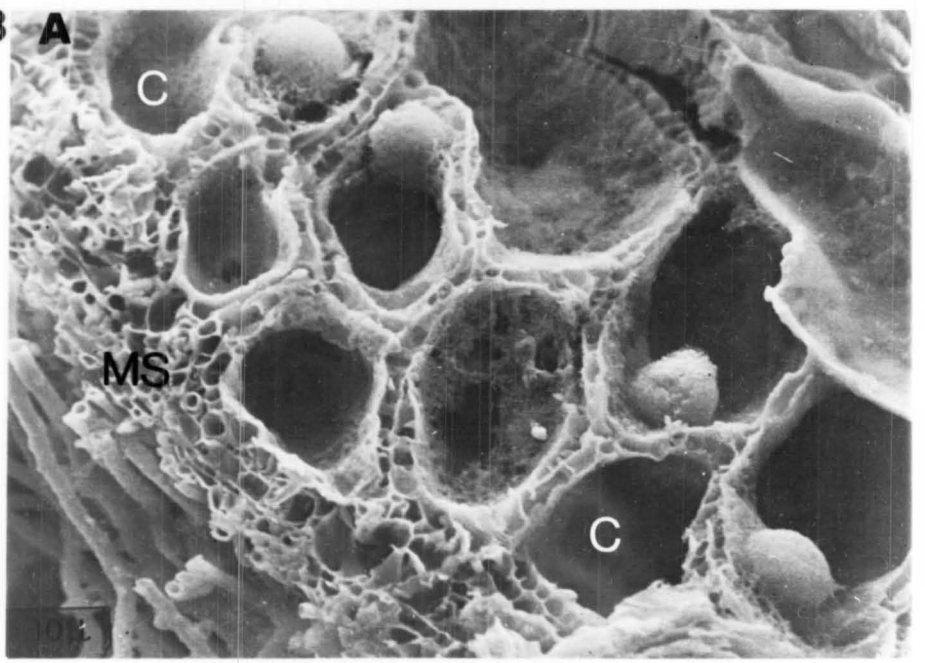
B The Hartig net seen in thin, transverse section (HN). Cortical cell (C) plasmalemma can be seen adjacent to the cell/hyphal wall (P).

The large granular bodies evident in both micrographs are similar to those noted previously adjacent to vascular tissue in the meristematic region. x1850

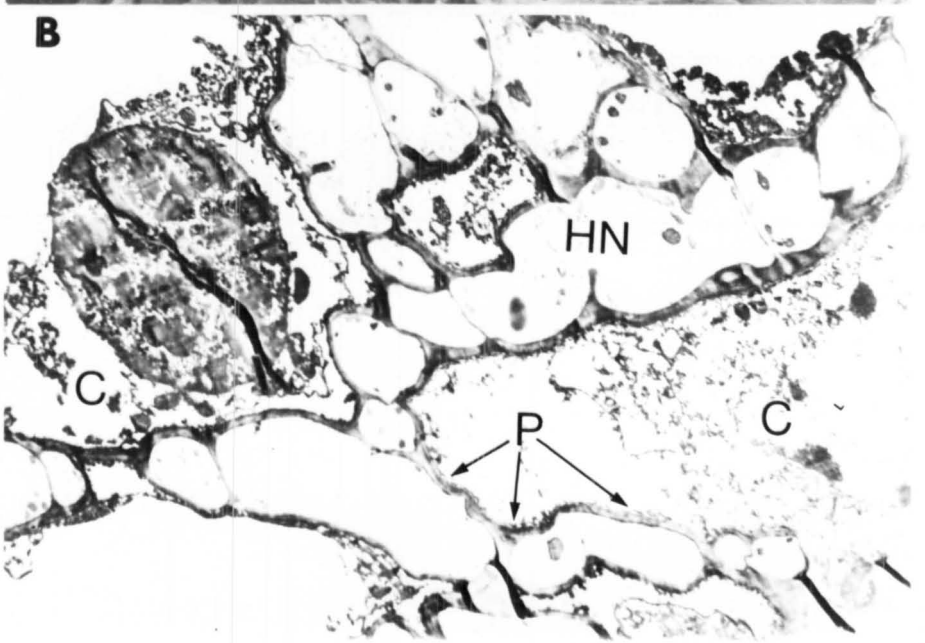
8-22



8-23 A



B



hyphal elements divide and expand between the cells (Fig. 8.23 A, B). Tannin deposits are present in the host cells (see also Hofsten, 1969) and the plasmalemma can be seen close to the host/fungal cell wall interface. The large granular bodies seen in both micrographs are of unknown composition. Details of Hartig element intrusion between cortical cells are seen in Fig. 8.24. A single hyphal layer is present in (A) and the effect of swelling in these elements is evident, causing the cell wall to bulge into the cell lumen. A typical two layered network is seen in (B) and the thin cell wall, which would presumably facilitate nutrient exchanges, is evident. The outline of underlying hyphae can be seen through the cell wall in Fig. 8.25 and the intricate nature of this network is shown in Fig. 8.26 and in Fig. 8.27.

As the fungal invasion proceeds, cells of the outer cortex commonly become filled with tannin (Fig. 8.28 A, B), a reaction thought to restrict colonization of the host by the fungus, or possibly associated with selective exclusion of non-mycorrhizal fungi (Marks and Foster, 1973; Ling-Lee et al. 1977). These cells, containing non-soluble polyphenols, eventually form a barrier embedded in the sheath and extending from the root tip to the root base, being broken at regular intervals by sheath hyphae passing into the cortex (Fig. 8.29). A similar barrier, formed by the endodermis, is thought to prevent vascular invasion.

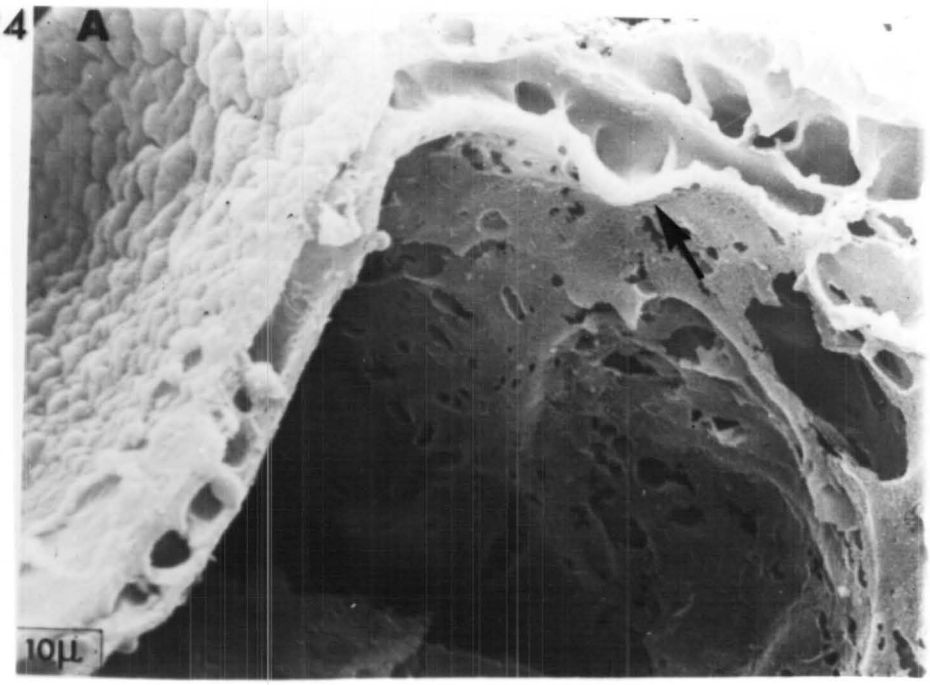
Deposition continues until the tannin cells show a uniform internal structure (Fig. 8.30 A, B) with no original

FIGURE 8.24 Hartig net element intrusion between cortical cells.

A Single layer intrusion - note the hyphal swelling (arrow). x650

B Two layered intrusion - note thin wall between cell and net. x900

8-24 A



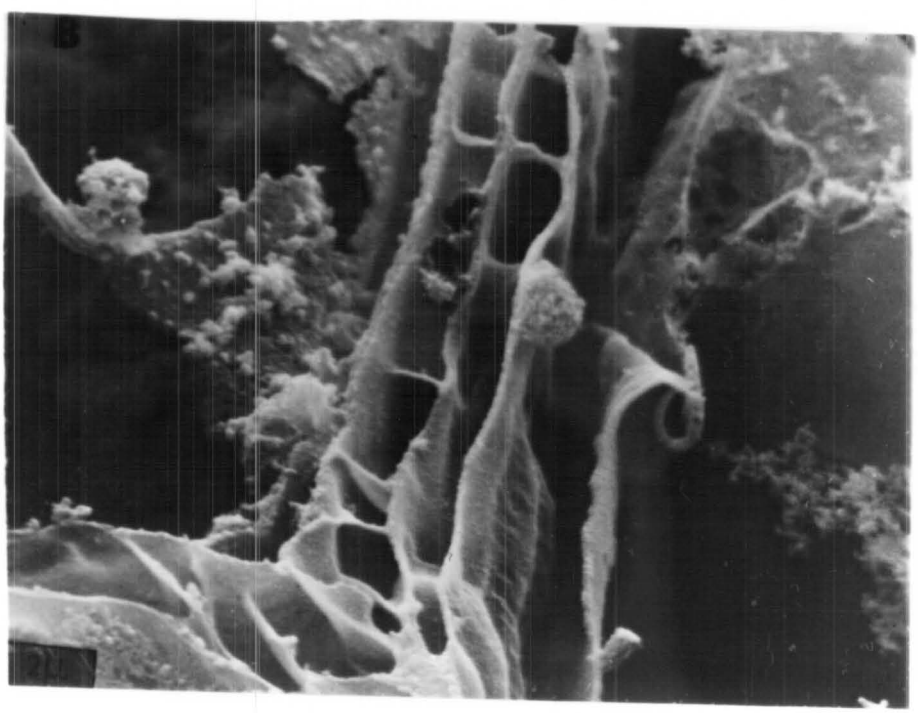


FIGURE 8.25 The Hartig net.

The net hyphae (HN) can be seen behind the cortical cell wall (CW). x670

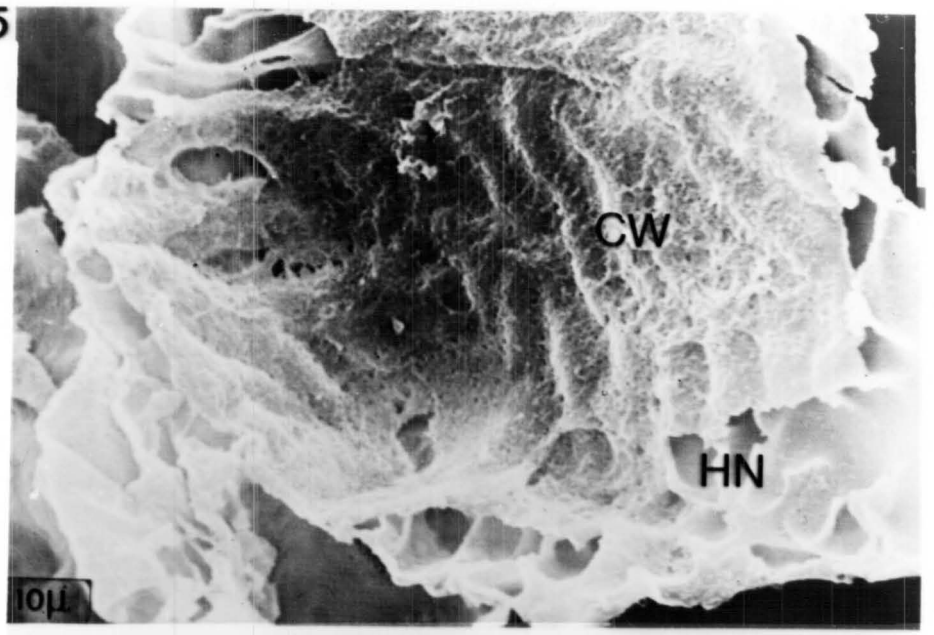
FIGURE 8.26 The Hartig net.

Oblique section of net hyphae (HN) showing their anastomosing nature. x650

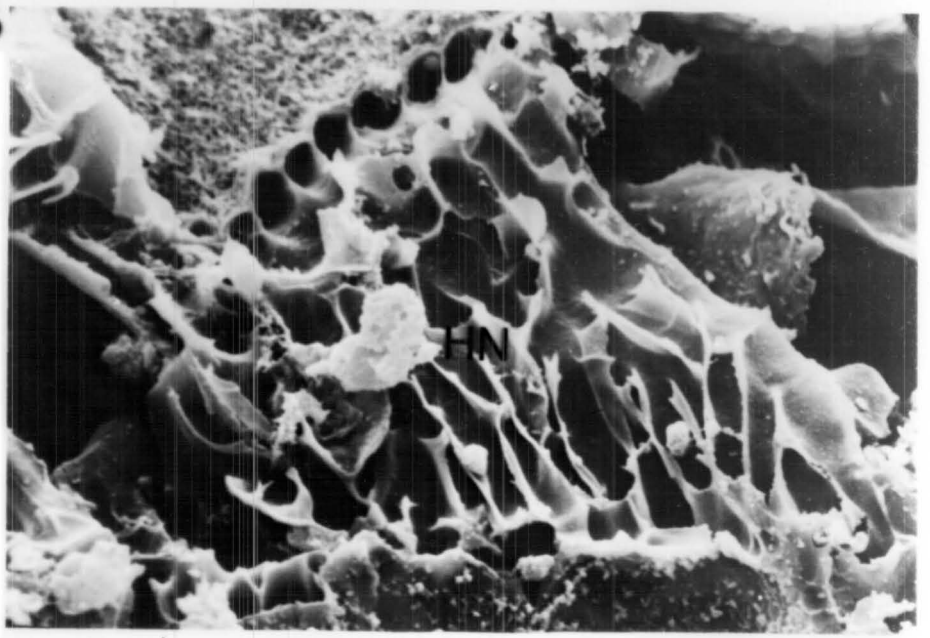
FIGURE 8.27 The Hartig net.

Median section of the network (HN) surrounding a cortical cell (C) showing the maze-like arrangement of the hyphae. x460

8-25



8-26



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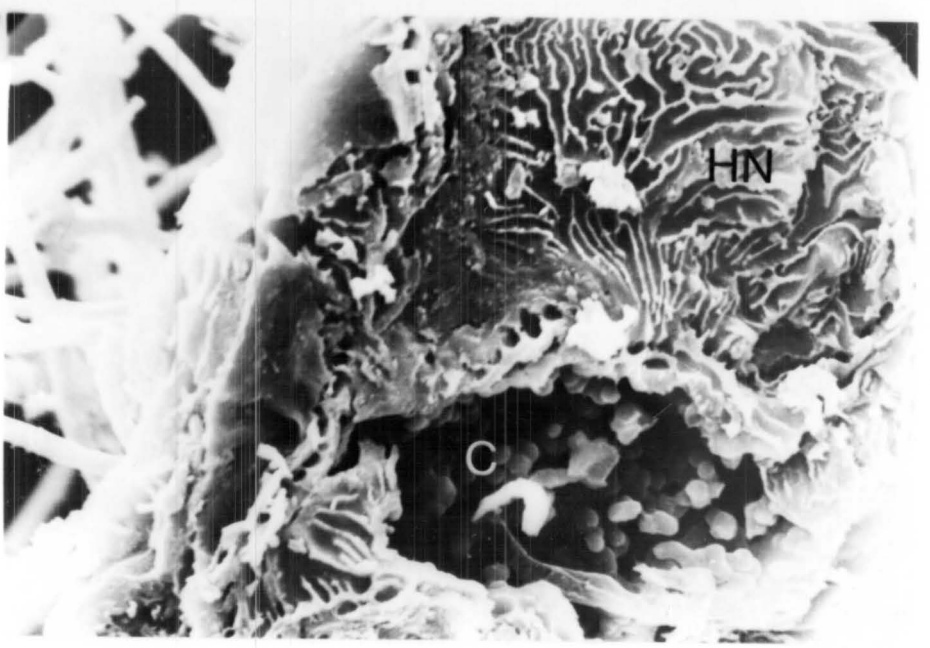
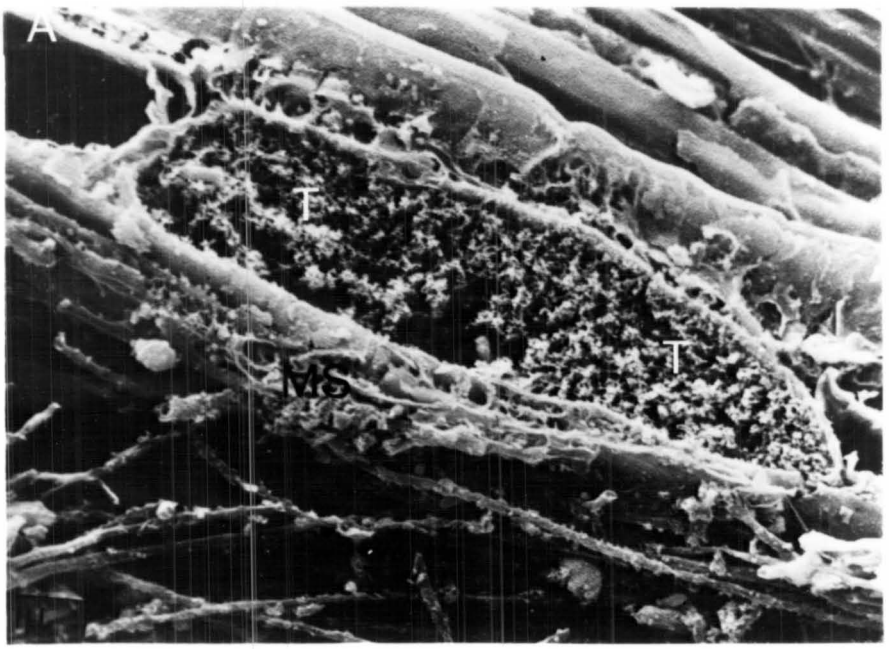


FIGURE 8.28 Tannin cell development.

A&B Cortical cells adjacent to the fungal sheath (MS) showing partial deposition of tannins (T).

A x380
B x700

8-28



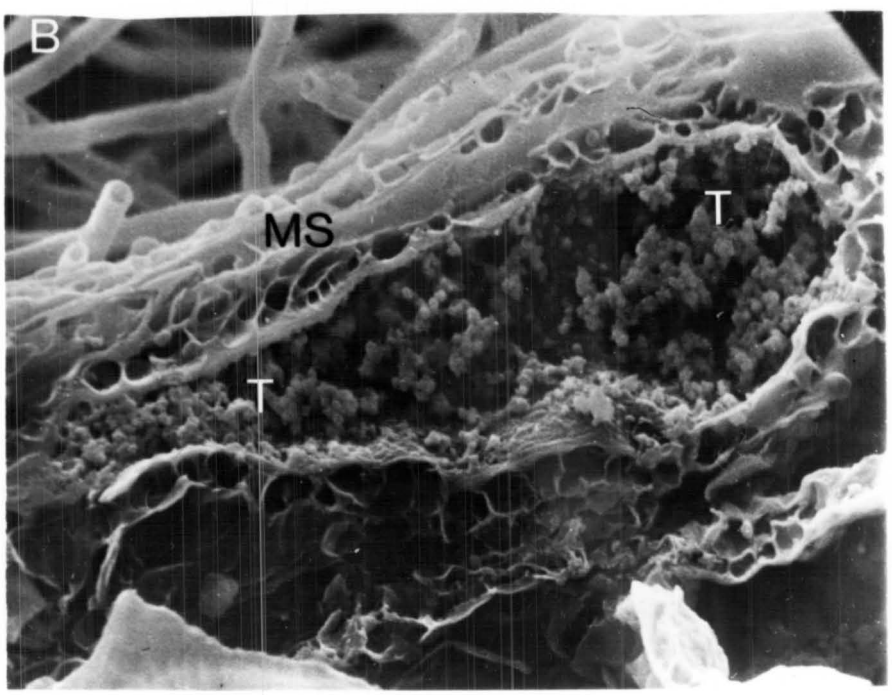


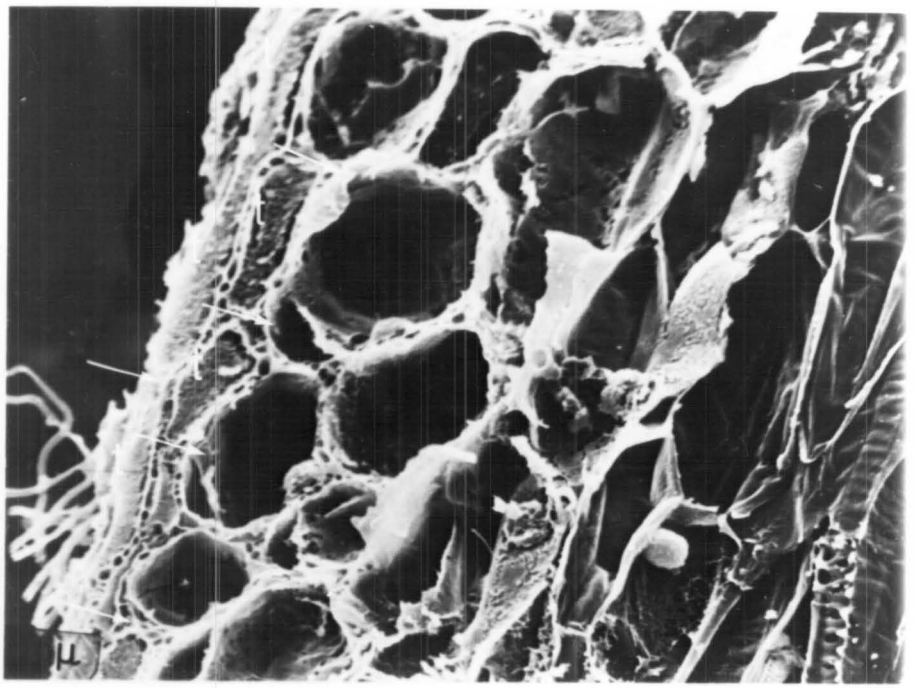
FIGURE 8.29 Tannin cell barrier.

The tannin cells (t) eventually form a barrier along the short root, broken at intervals by sheath hyphae passing into the cortex (arrows). x390

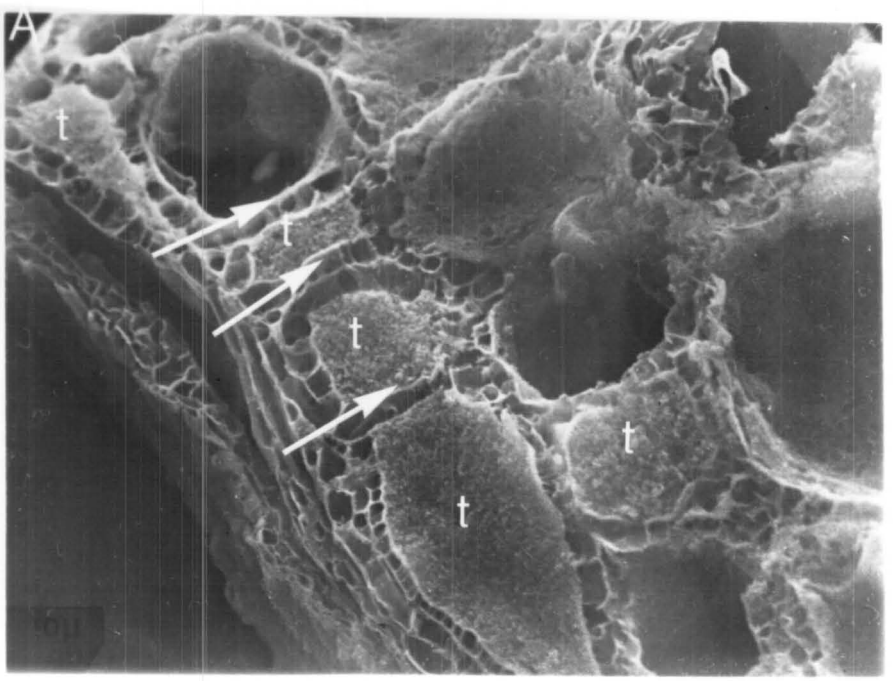
FIGURE 8.30 Tannin cell barrier.

A Deposition in the tannin cells (t) continues until they show a uniform internal structure. Again hyphae can be discerned passing between the tannin cells into the cortex (arrows). x420

8-29



8-30



cell contents being visible (Marks and Foster, 1973). The cell wall between sheath and tannin cell is thickened (B) and polyphenols are also deposited between hyphae of the sheath. An unusual feature, noted in some roots, was the presence of an occasional cell with a highly vacuolate appearance in the outer cortex or tannin layer (Fig. 8.31).

8.3.3 'Vesicular Body' Formation in Mycorrhizal Roots

This study revealed novel 'vesicular bodies', present in many cortical cells of mycorrhizal roots (Fig 8.32). A few were noted in cells of control, non-infected, roots but were greatly reduced in numbers. The 'vesicular bodies' were always found in close association with the Hartig net in cortical cells near the sheath and the tannin cell layer (Fig. 8.33). They were occasionally noted deeper in cortical and vascular tissue (Fig. 8.34). Those present in vascular tissue may be hollow as they appear to be collapsed in some cases (Fig. 8.35).

In most cells the 'vesicular bodies' seem to be attached to a membrane-like structure which may be part of the cell wall. This layer has separated from the cell wall in Fig. 8.36 and 'vesicular bodies' can be seen through it. The prevalence of these structures in mycorrhizal roots is extremely varied. Some roots have few cells containing the inclusions (Fig. 8.37) but in others they are prolific (see Fig. 8.34) and there is often a large variation in density and size of 'vesicular bodies' between adjacent cells of the same root (Fig. 8.38). Two types can be distinguished.

FIGURE 8.30 Tannin cell barrier.

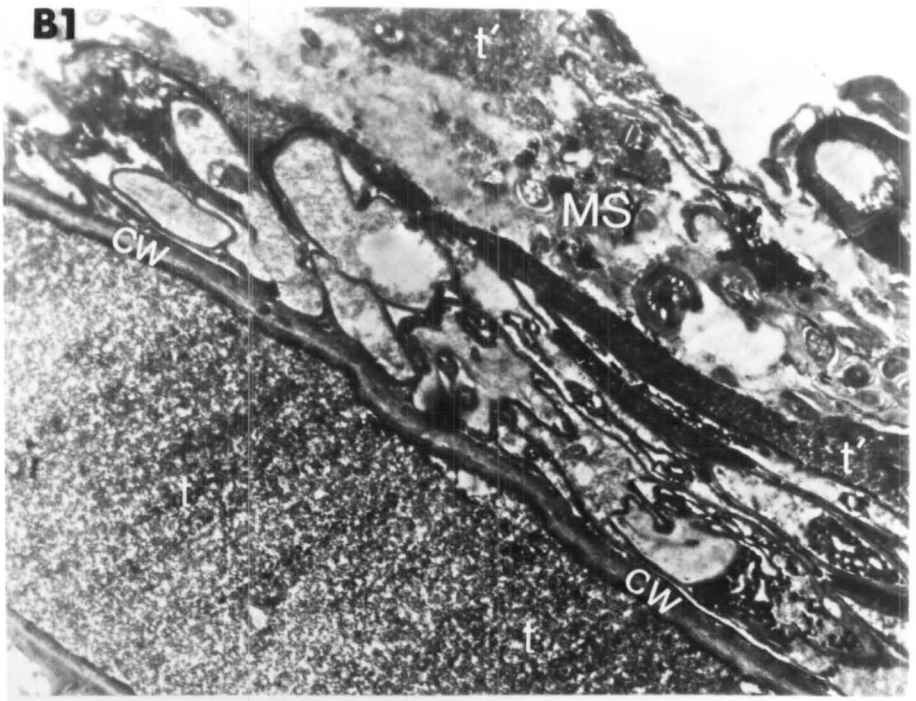
B1 Section across sheath (MS) and a tannin cell (t). Note thickness of cell/hyphal wall (CW) between sheath hyphae and the tannin cell and deposition of tannins between sheath elements (t'). x2500

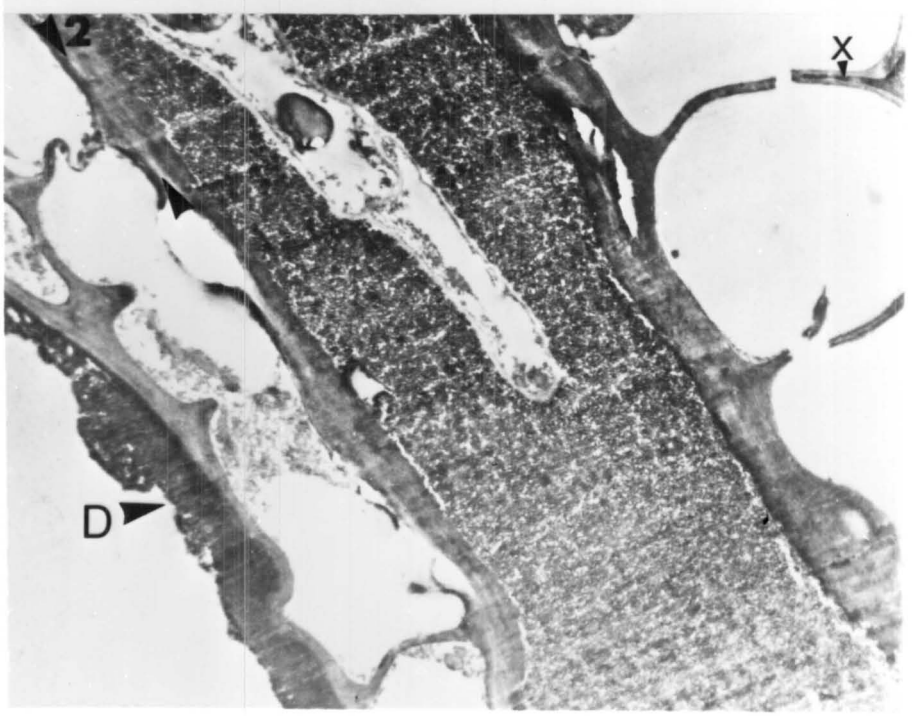
B2 Tannin cell/hyphal junction. Hyphal walls appear to be tripartite in nature (x) but it is difficult to discerne between the fungal cell wall and that of the host (arrows). Depositions have occurred on the adjacent cortical cell wall (D). x3000

FIGURE 8.31 Vacuolate cell.

These cells, with their unusual appearance, were infrequently found in the outer cortical layers. x700

8-30 **B1**





8-31

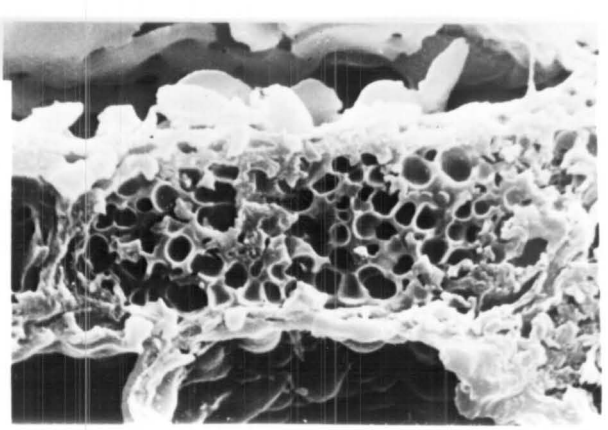


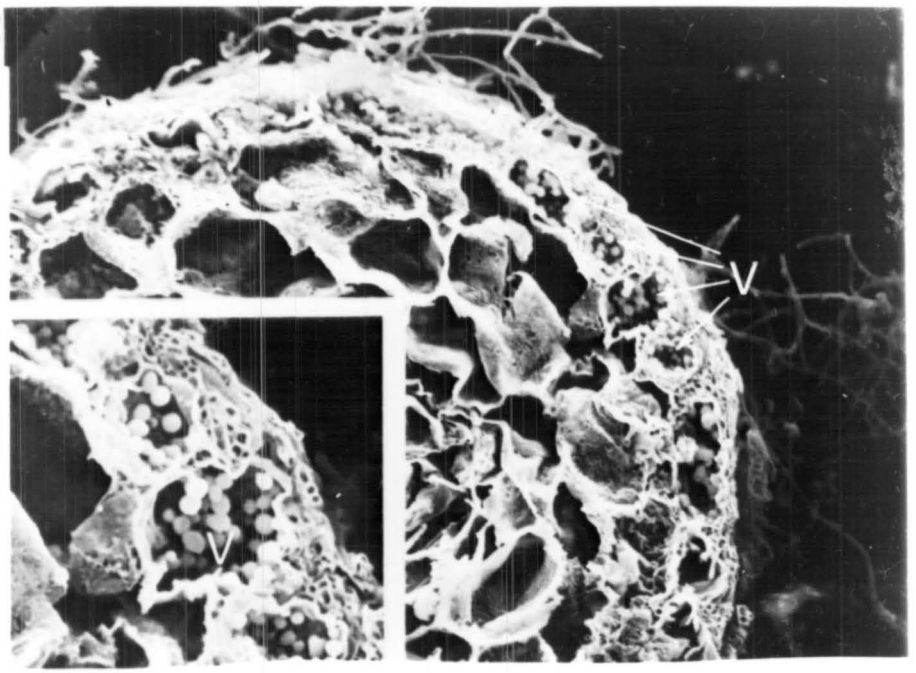
FIGURE 8.32 'Vesiculate bodies' in mycorrhizal roots.

These bodies (V) are often found in outer cortical cells of mycorrhizal short roots and are closely associated with the sheath and Hartig net (inset). x160

FIGURE 8.33 'Vesiculate bodies'.

The association of the bodies (V) with the sheath (MS) and Hartig net (HN) is evident. A tannin cell is also shown (t). x630

8-32



8-33

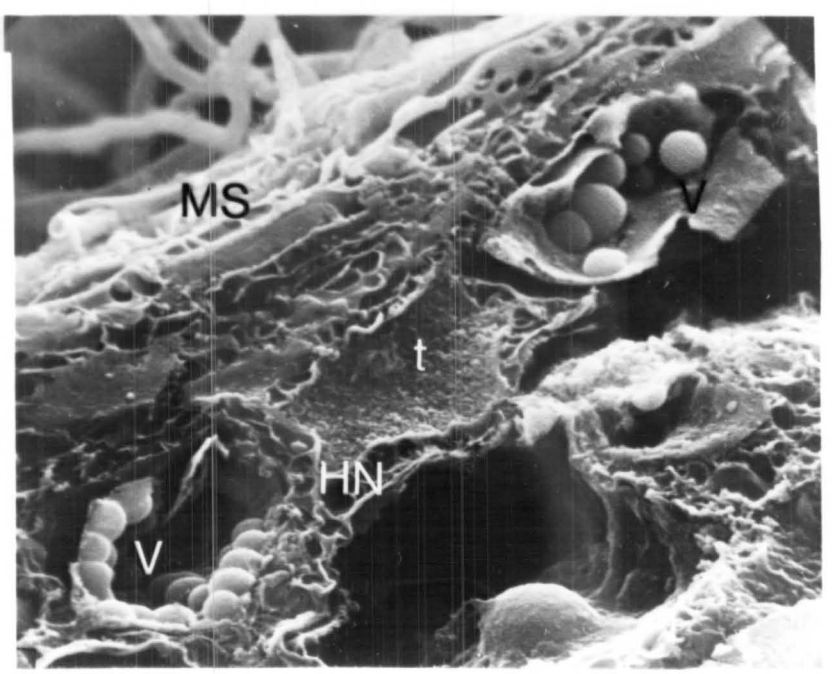


FIGURE 8.34 'Vesicular bodies' found in deeper cortical tissue.

In this case they are seen in cortical cells close to vascular tissue and their size appears to decrease from the peripheral cortex (lower) to the internal cortex (upper).

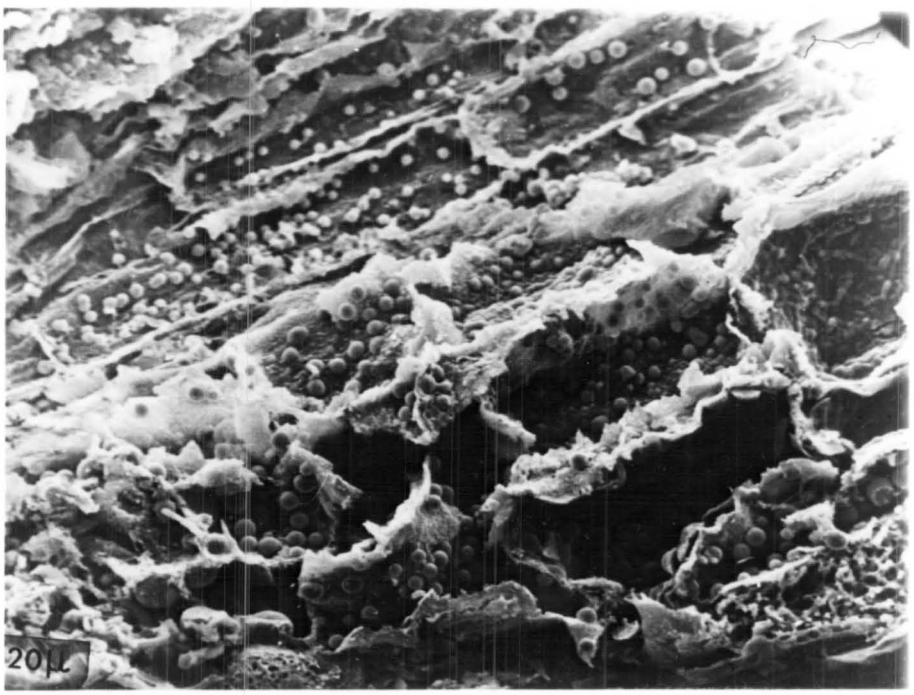
This micrograph shows the prolific nature of the 'vesicular bodies' found in some roots. x300

FIGURE 8.35 'Vesicular bodies' (V) seen in vascular tissue.

These appear more eliptical in shape and may be hollow as a certain amount of collapsing is evident (as arrowed).

x1700

8-34



8-35

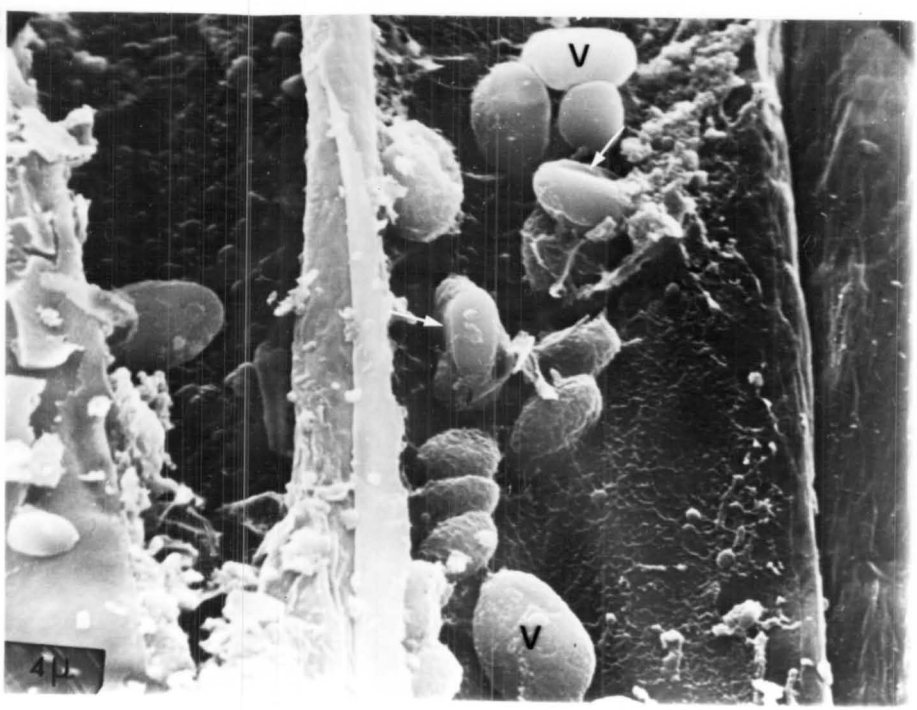
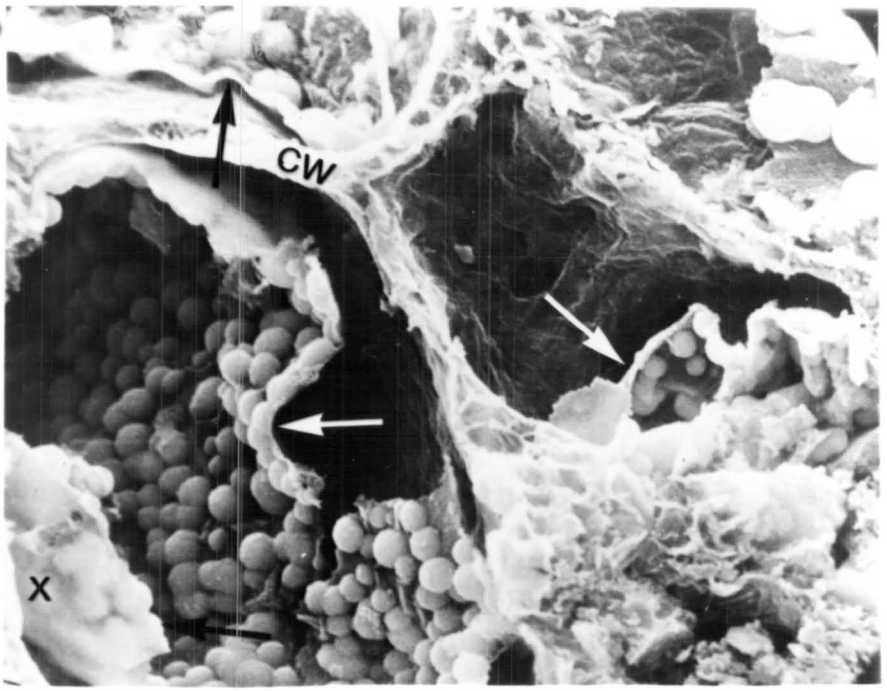


FIGURE 8.36 'Vesicular body' attachment to a membranous structure.

This membrane (arrows) may be part of the cell wall (CW) and 'vesicular bodies' are visible through it in places(X), indicating that it is very thin. x600

FIGURE 8.37 In some root sections very few cells have 'vesicular bodies'. This was the only cell with 'vesicular bodies' noted in a particular section, but shows their close relationship to the Hartig net (HN) and the membranous structure (arrows). x420

8-36



8-37

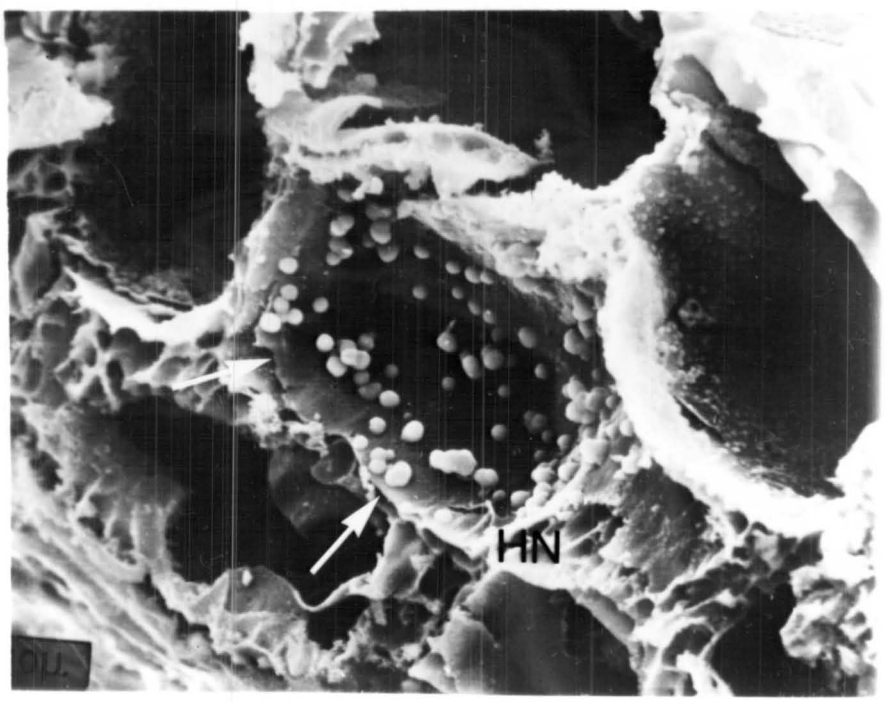
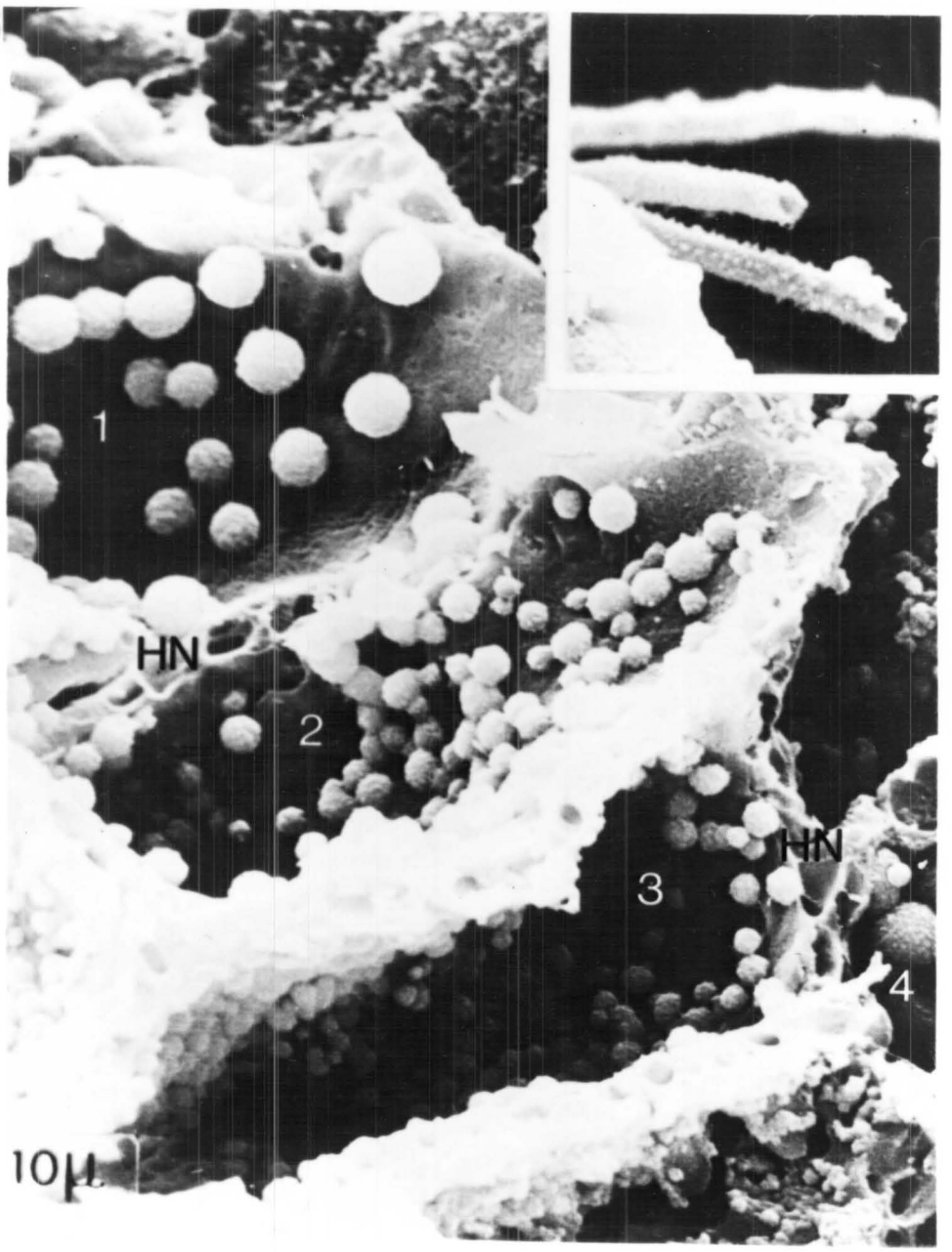


FIGURE 8.38 Prevalence of 'vesicular bodies' in root sections.

In this root section, showing prolific numbers of 'vesicular bodies', variations in both density and size are also evident between adjacent cortical cells (1, 2, 3, 4). Fewer large bodies are found (these being $\geqslant 5\mu m$ size) but many of the smaller sized bodies are seen (2-4 μm). Hartig net elements (HN) are also shown. Note the resemblance between the surface features of the papillated hyphae (inset) and of the 'vesicular bodies'. x1200

8-38



The predominant type, shown in Fig. 8.39, have a rough surface, in contrast to the less frequently found smooth type (Fig. 8.40). The 'vesicular bodies' range in size from 2 μm to 6 μm in diameter and fewer of the large type are found per cell. Transitional forms are also found. Most vesicular bodies are spherical in outline (Fig. 8.41 A, B) and have slightly roughened surfaces but there is evidence for coalescence of adjacent vesicles. The wide variation in size and form that is seen even within a single cell is shown in Fig. 8.42. With increased size and coalescence of the 'vesicular bodies', they are occasionally found to cover the entire inner surface of the cell wall. 'Vesicular bodies' nearing this stage of development can be see in Fig. 8.43. Many vesicles have coalesced and they have a rough appearance (very similar to the papillated surface of the fungal hyphae - Fig. 8.38). Some have also been detached from the cell wall but it is difficult to determine whether they are vacuolate or uniform in composition. Those seen in Fig. 8.44 do appear to be hollow, opening into the Hartig net behind the cell wall. Details of coalescence and growth of the 'vesicular bodies' are evident at increased magnification (Fig. 8.45). Their close relationship to the Hartig net is clearly visible in Fig. 8.46. Continuity of some large vesicles with the fungal hyphal network is evident in Fig. 8.47, in this case where a portion of one vesicle's wall has been removed (arrow).

These 'vesicular bodies' appear to be consistent features of P. mugo mycorrhizal roots, although their

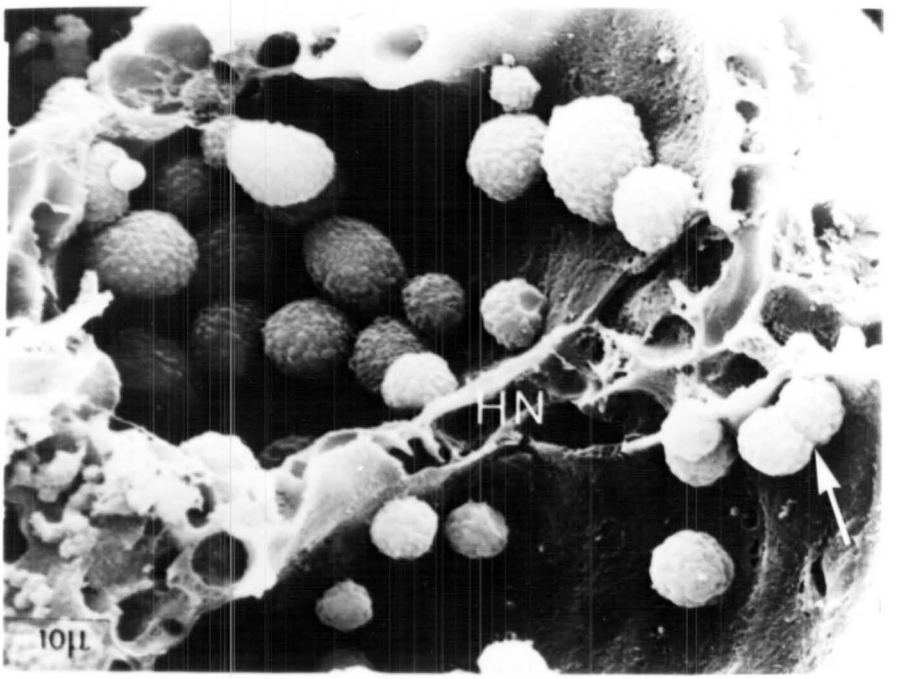
FIGURE 8.39 Rough 'vesicular bodies'.

This type of body was most commonly found in the mycorrhizal root sections. Note the presence of the Hartig net (HN) and the apparent coalescence of some 'vesicular bodies' (arrow). x1800

FIGURE 8.40 Smooth 'vesicular bodies'.

Less frequently found, these also show a range of size and coalescence (arrows). The Hartig net (HN) is also shown. x1840

8-39



8-40

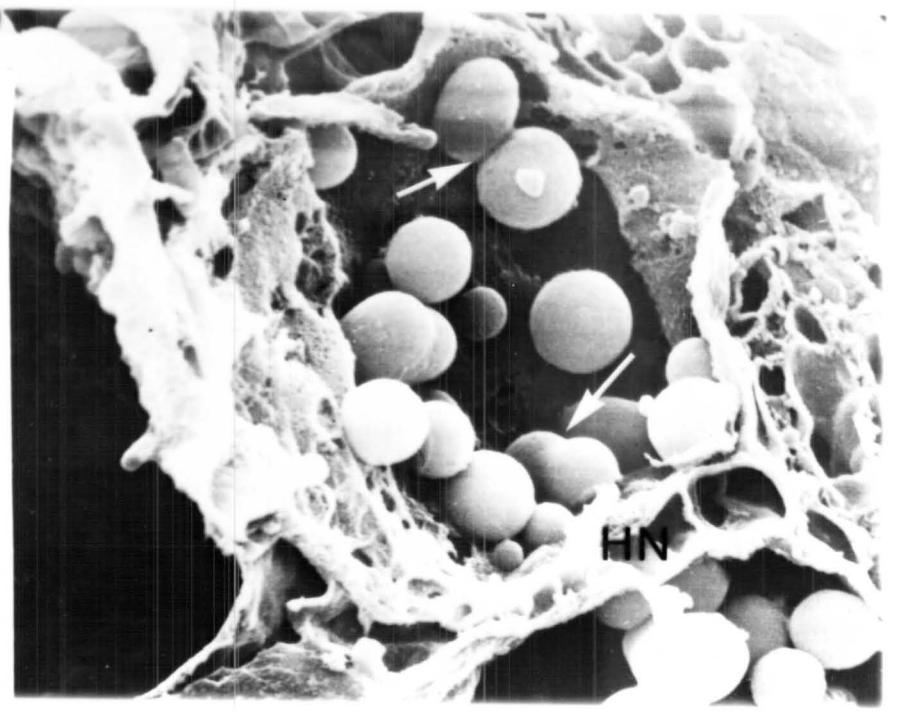


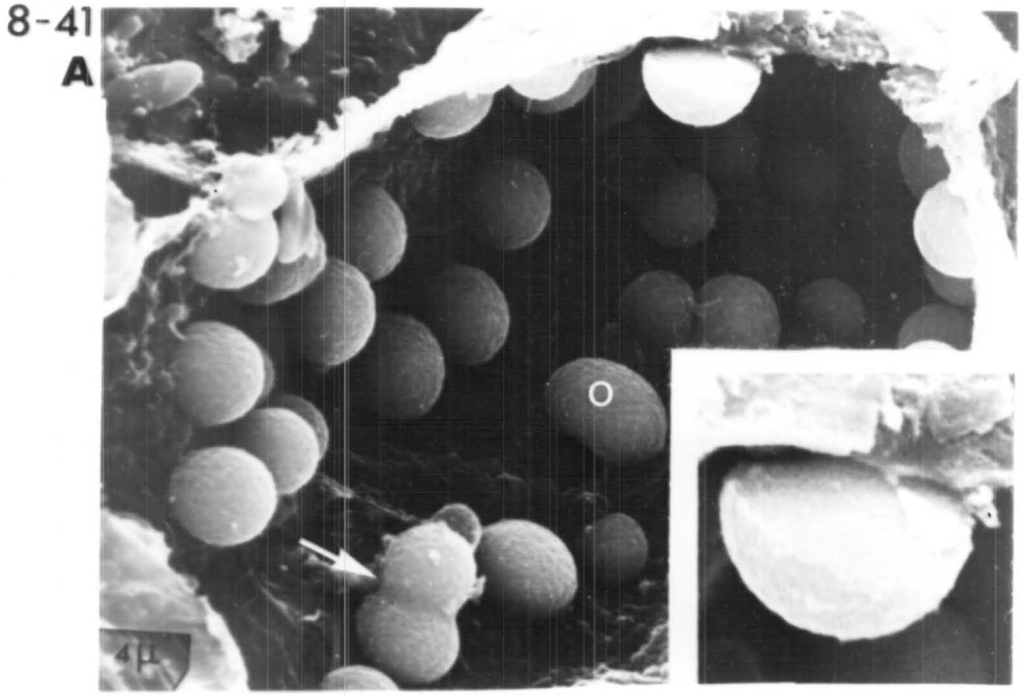
FIGURE 8.41 Transitional forms of 'vesicular body'.

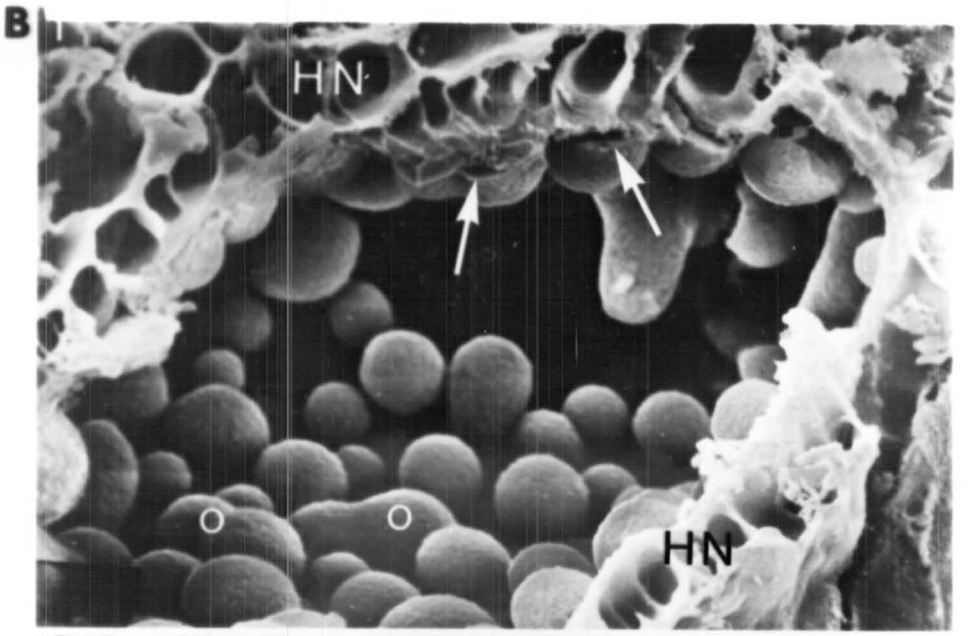
A The 'vesicular bodies' shown have surface features transitional between the rough and smooth types shown previously (8.39, 40). Coalescence is evident (arrow) and the spherical shape of some is altered (o). Note the 'vesicular body' at top right and inset - the underside is exposed and it would appear to be an encrusted growth on the cell wall.

x1700
Inset: x3380

B1&2 Surface features of the 'vesicular bodies' are again transitional and the shape of many is no longer spherical (o). The Hartig net is evident (HN) and some bodies have become detached from the cell wall (arrows). x1700

8-41





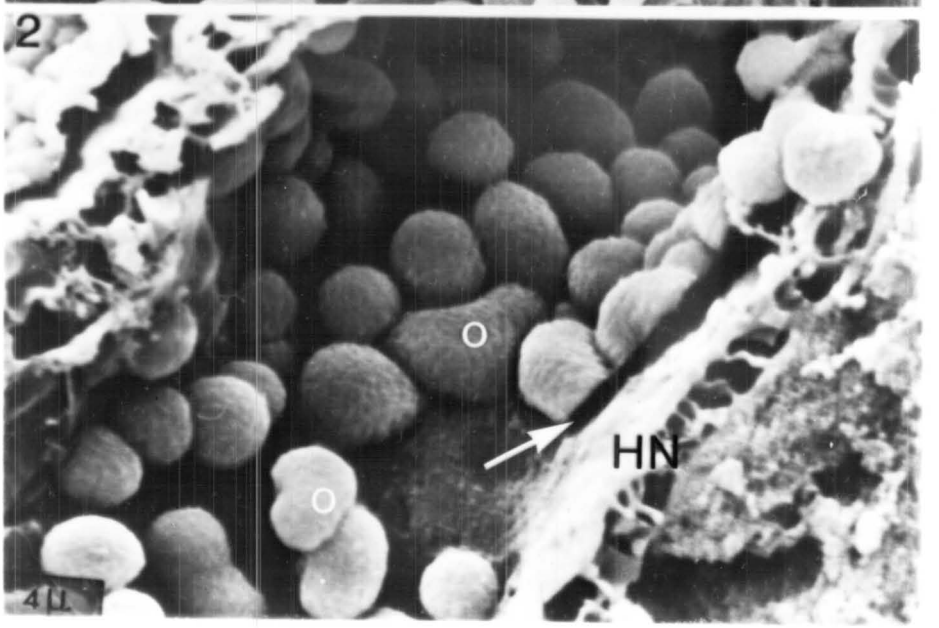


FIGURE 8.42 Variation in 'vesicular bodies' within a cell.

'Vesicular bodies' of several sizes are present in this cell and coalescence has occurred (arrow). x1900

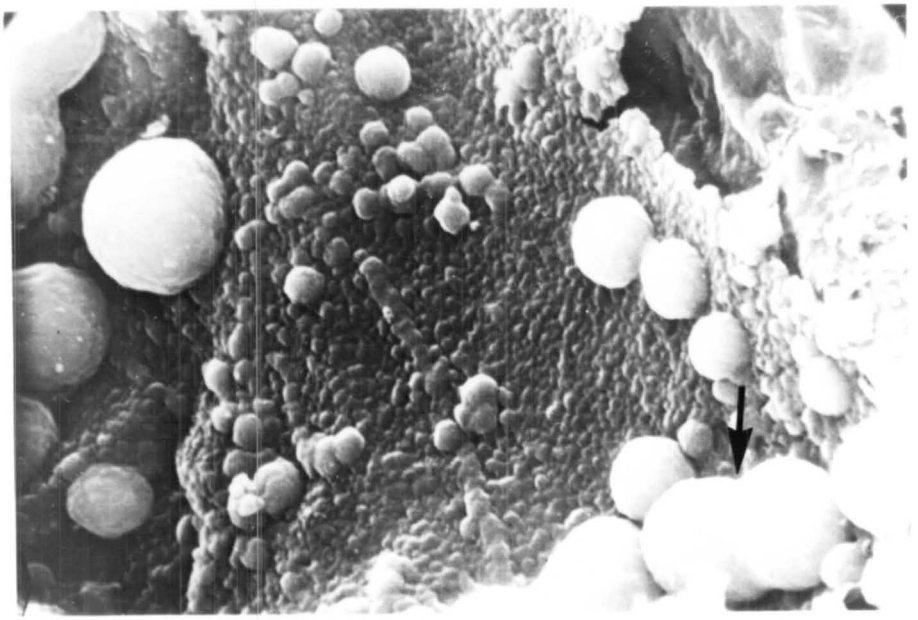
FIGURE 8.43 Coalescence of 'vesicular bodies'.

More extensive joining of the bodies is evident in this micrograph (o) and a large area of the cell wall is gradually becoming covered. Note the Hartig net (HN). x1650

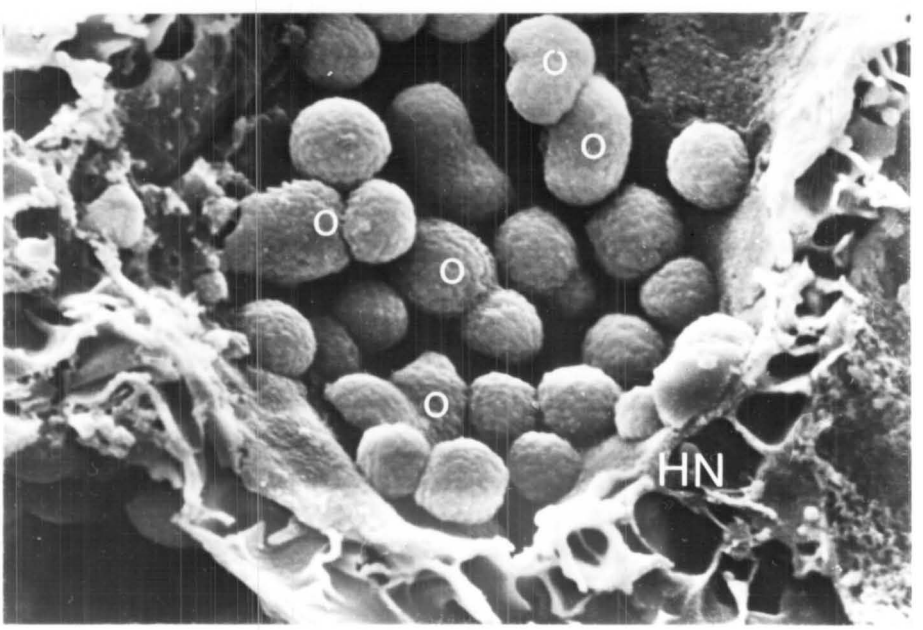
FIGURE 8.44 Hollow 'vesiculate body'.

It is difficult to determine whether the bodies are vacuolate or uniform in composition, but one shown here appears to open into the Hartig net (HN) behind the cell wall. x1500

8-42



8-43



8-44

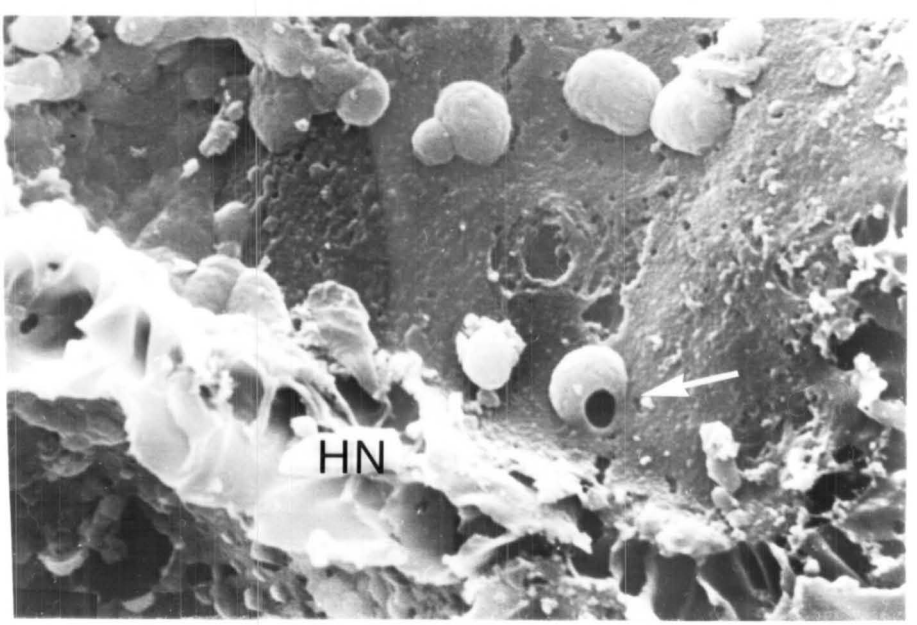


FIGURE 8.45 Coalescence of 'vesicular bodies'.

A Further detail of this process is shown. A large 'vesicular body' appears to be engulfing others on the cell wall (O) and others are at various stages of coalescence. Note the body at x which has been partially sectioned and appears to be a type of encrustation on the cell wall. x2100

B Three 'vesicular bodies' showing different stages of merging (arrows). x8600

8-45

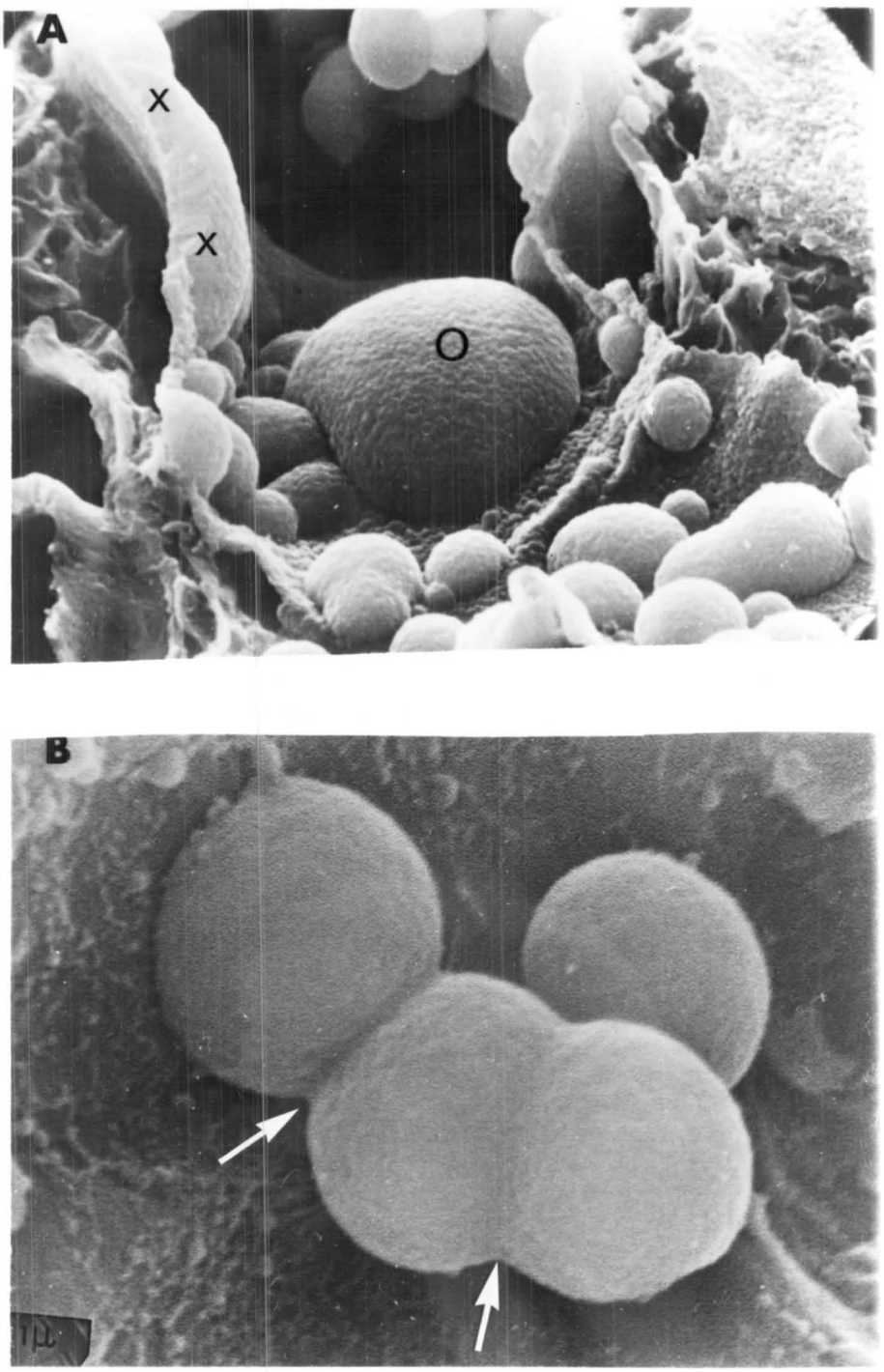


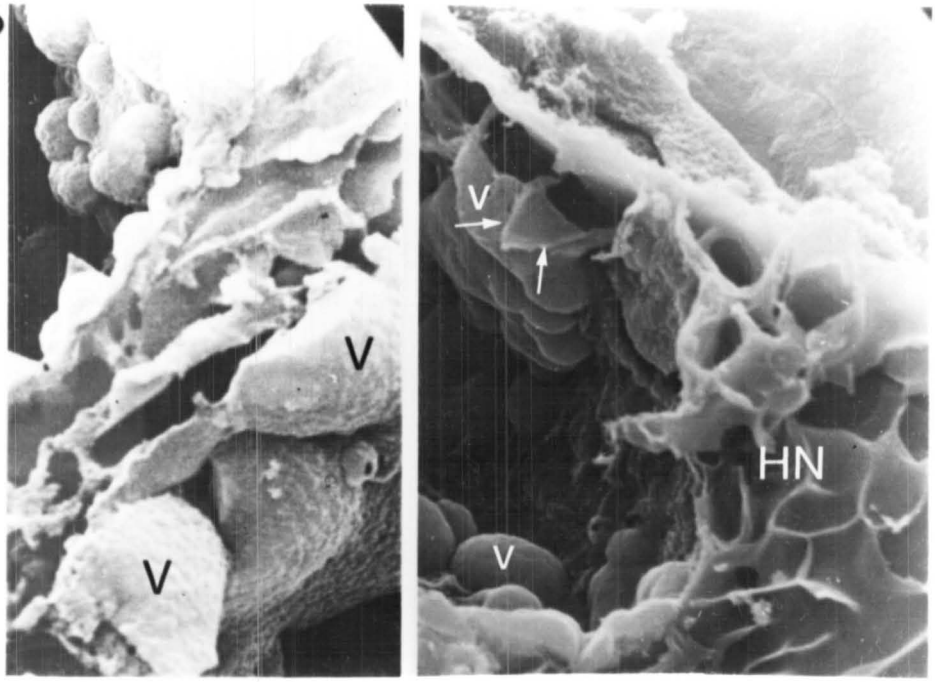
FIGURE 8.46 'Vesicular body' relationship to the Hartig net.

In both views shown the 'vesicular bodies' (V) are found in close apposition to the Hartig net (HN). Partial sectioning of a 'vesicular body' (arrow) reveals apparent continuity with the fungal hyphae comprising the net. x1840
x1440

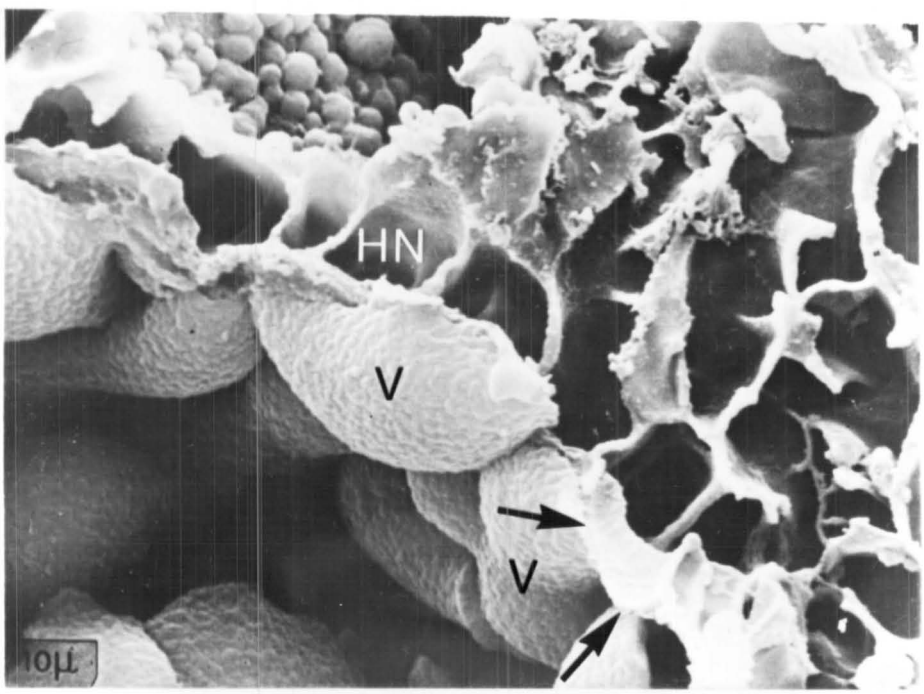
FIGURE 8.47 'Vesicular body' relationship to the Hartig net.

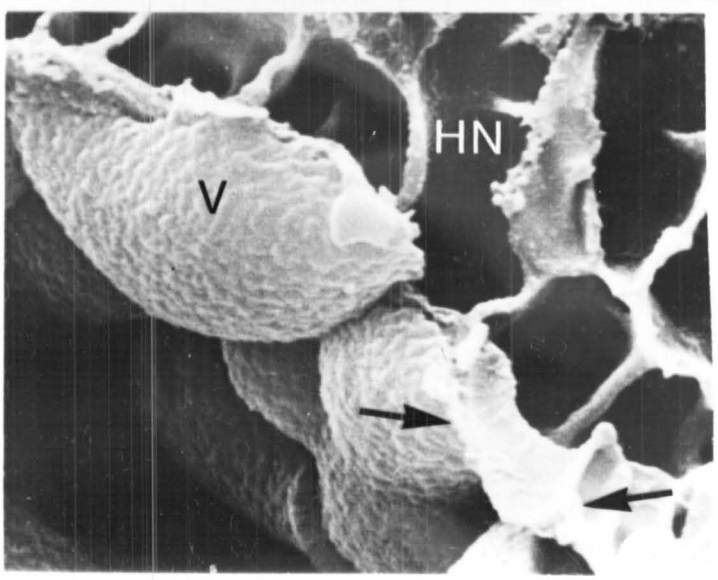
Large, irregular shaped, rough 'vesicular bodies' (V) are shown covering a large portion of the cell wall. They are clearly related to the Hartig net (HN) as seen where partial sectioning has revealed the internal structure of a 'vesicular body'. This is shown in more detail in the lower micrograph.
x1870
x2430

8-46



8-47





occurrence within the root is irregular. Following the S.E.M. study, root sections were prepared by freezing microtomy, maintaining them in their natural state, and observed. These also showed the presence of swellings and vesicles in similar situations to those described above (Fig. 8.48). Many areas of the Hartig net show similar swellings to those reported by Mikola (1965) in ectendotrophic mycorrhizas (Fig. 8.49). In some of the cortical cells the 'vesiculate bodies' could be seen on the inner wall surfaces (Fig. 8.50), again showing a wide range of size and shape (Fig. 8.51). The vesicles can fill much of the cell lumen (Fig. 8.52) and resemble tyloses found in some plants exhibiting resistant reactions to pathogens.

8.3.4 Discussion

This study shows the complex nature of mycorrhizal infection and development in P. mugo. It appears that most mycorrhizal short roots are infected at an early stage, before or shortly after their emergence from the mother root. Laterals developing as long roots, however, may also become mycorrhizal and both these and the short roots exhibit cessation of elongation after this occurs. Only when elongation has ceased can the hyphae form a complete sheath over the root. With the S. luteus/P. mugo association only the middle lamella between host cells is degraded to allow fungal colonization and with consequent swelling of net hyphae, the entire mycorrhizal root becomes swollen, often having a translucent appearance. The process of infection is summarised in Fig. 8.53.

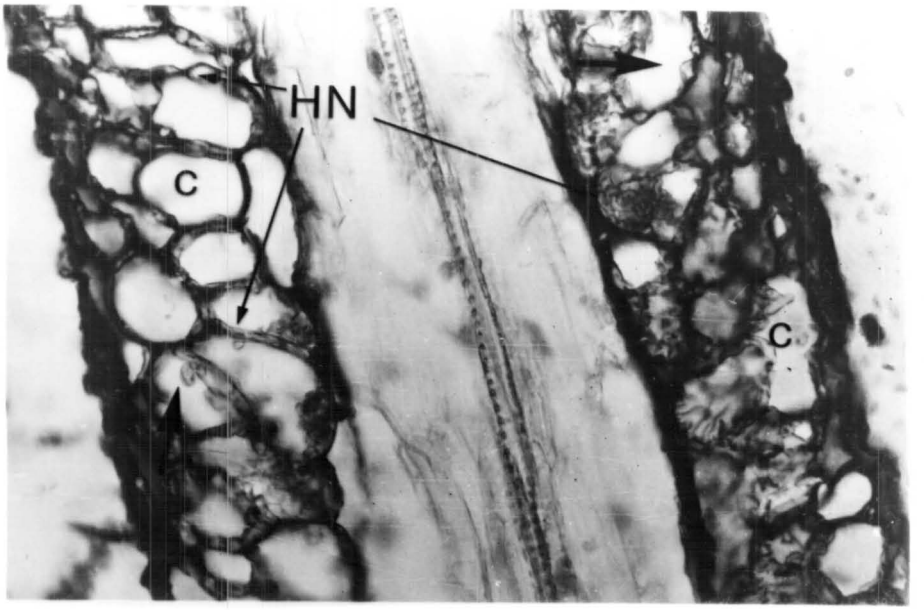
FIGURE 8.48 Whole root section prepared by freezing microtomy.

The extent of Hartig net (HN) development between the cortical cells (C) is evident. This often forms swollen protuberances into the cortical cells. (arrows). x200

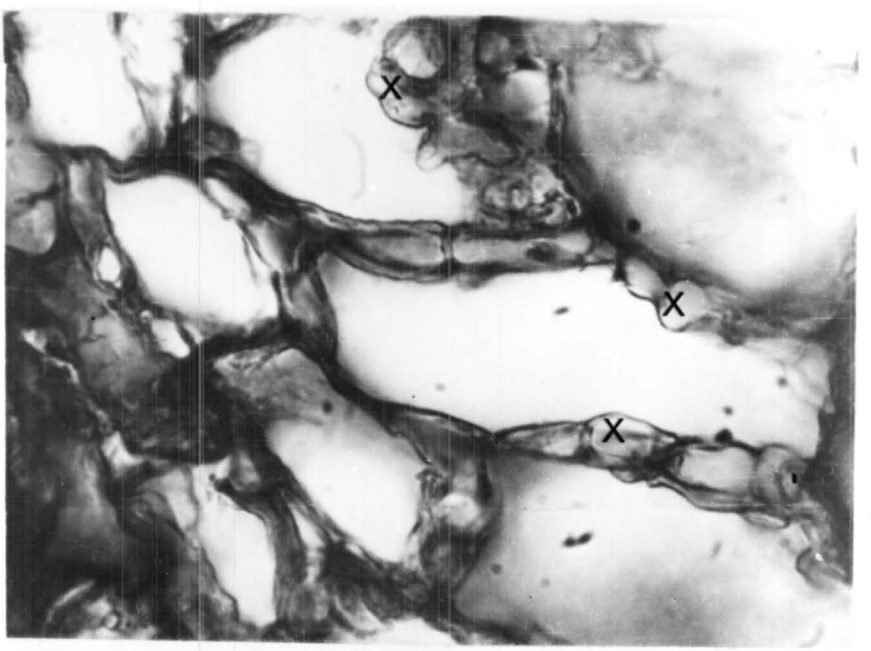
FIGURE 8.49 Root sections showing ectendotrophic-like swellings.

Swollen Hartig net elements (X) were often found but the three-dimensional appearance of them is not clear in thin section. Note the presence of sectioned Hartig net in some cells (cf 8.27) (arrows). x800
x200

8-48



8-49



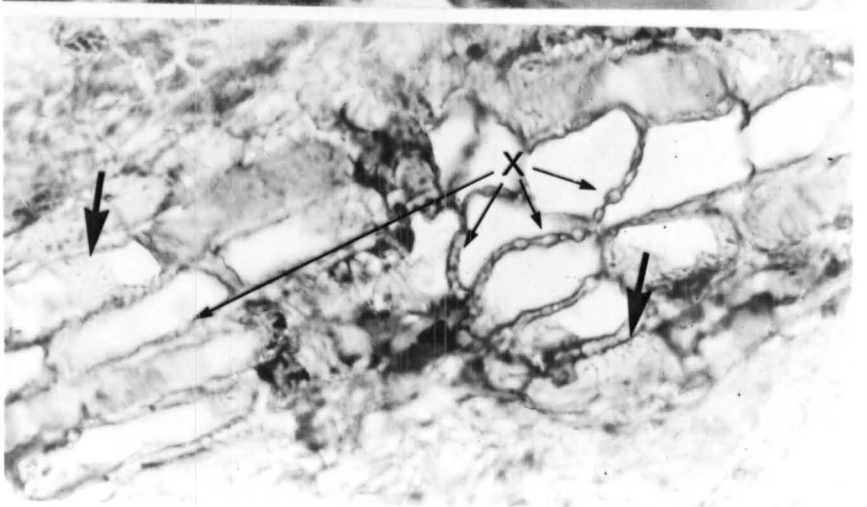


FIGURE 8.50 Cortical cell showing 'vesiculate bodies' (V).

A range of size and density of the 'vesiculate bodies' was again evident in root sections prepared by freezing microtomy. x800

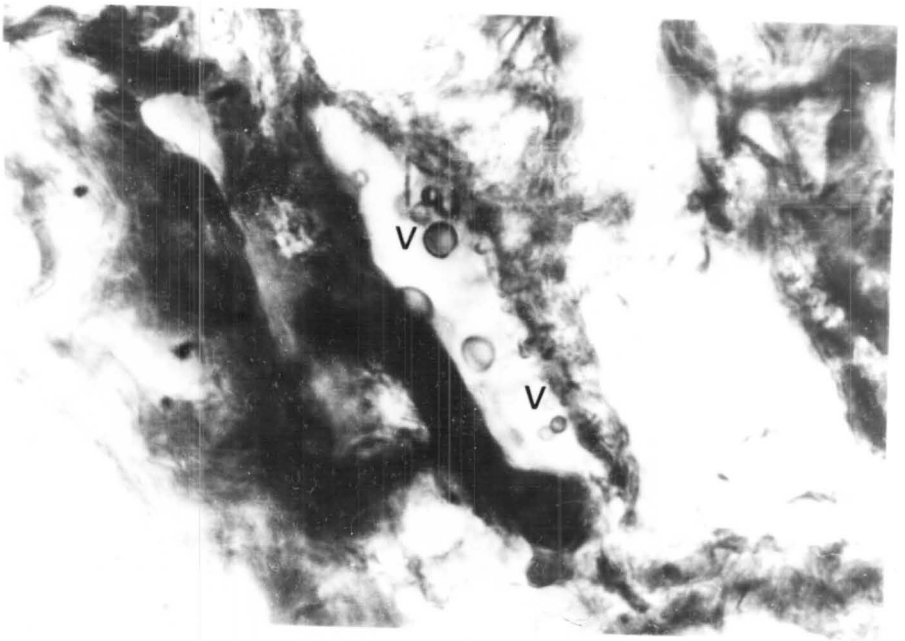
FIGURE 8.51 'Vesiculate bodies' in adjacent cells (1, 2).

Size and density variation is apparent (see Fig. 8.38). x800

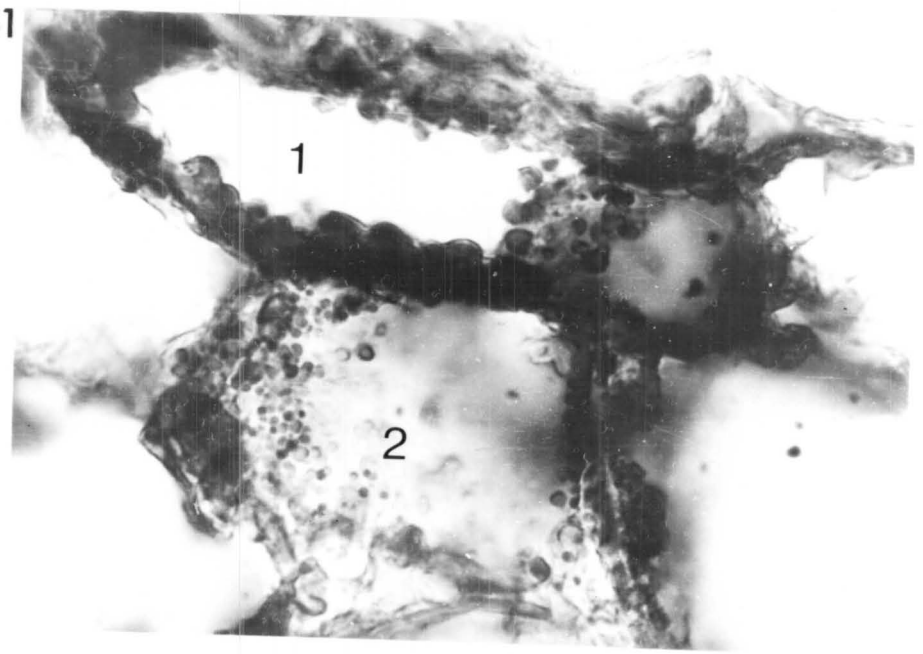
FIGURE 8.52 'Vesiculate bodies' apparently filling much of the cell lumen and resembling tyloses((T).

These are probably similar to the 'vesicular bodies' shown in Fig. 8.47 on the cell wall, but appear to lie in the lumen because of the shallow depth of field of the light microscope. x800

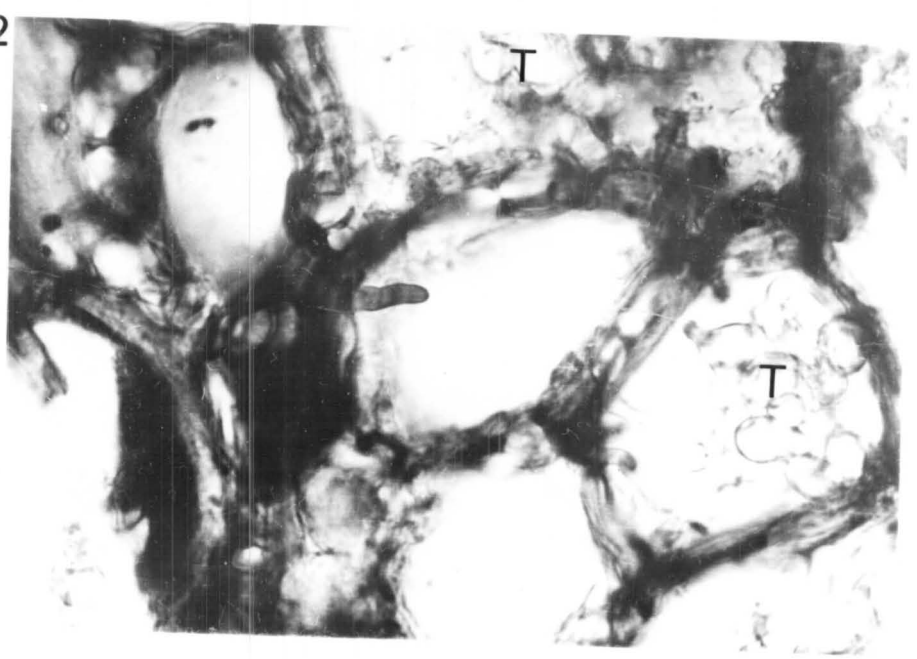
8-50



8-51

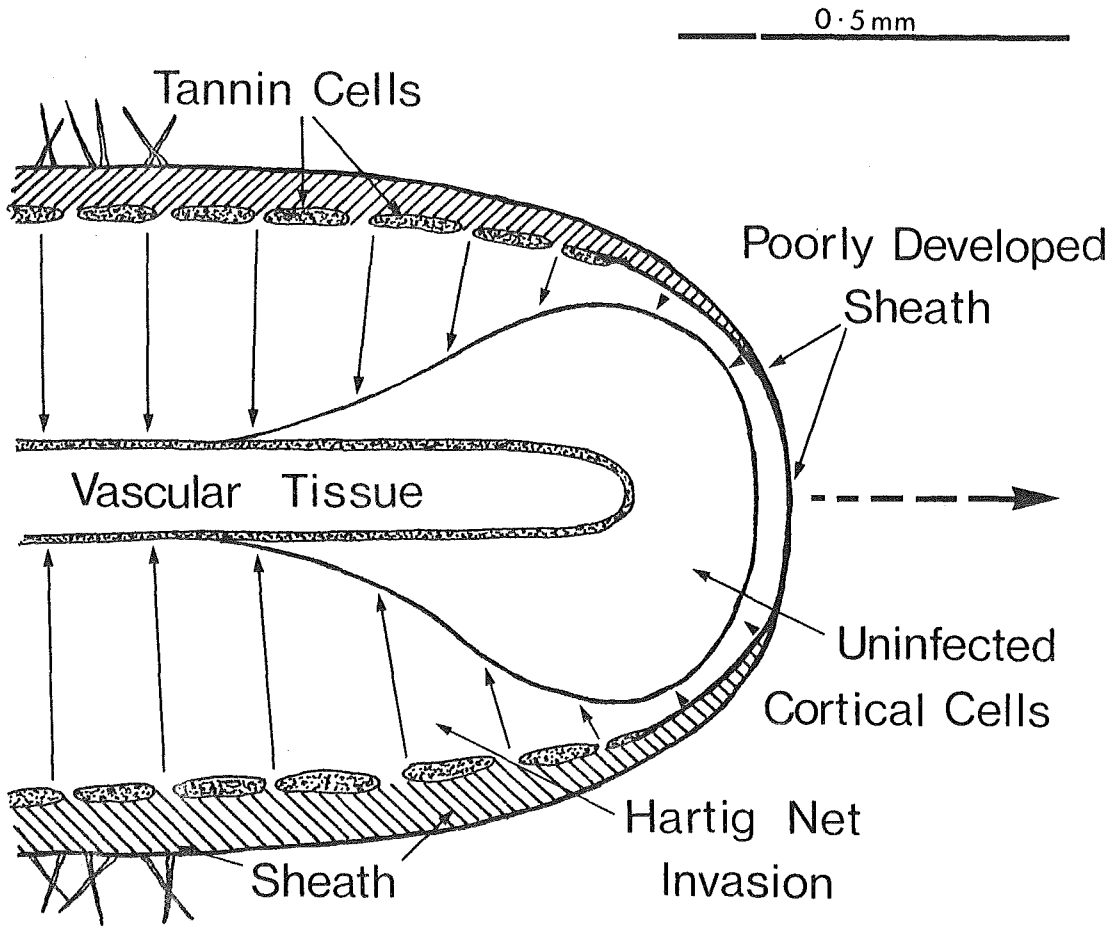


8-52

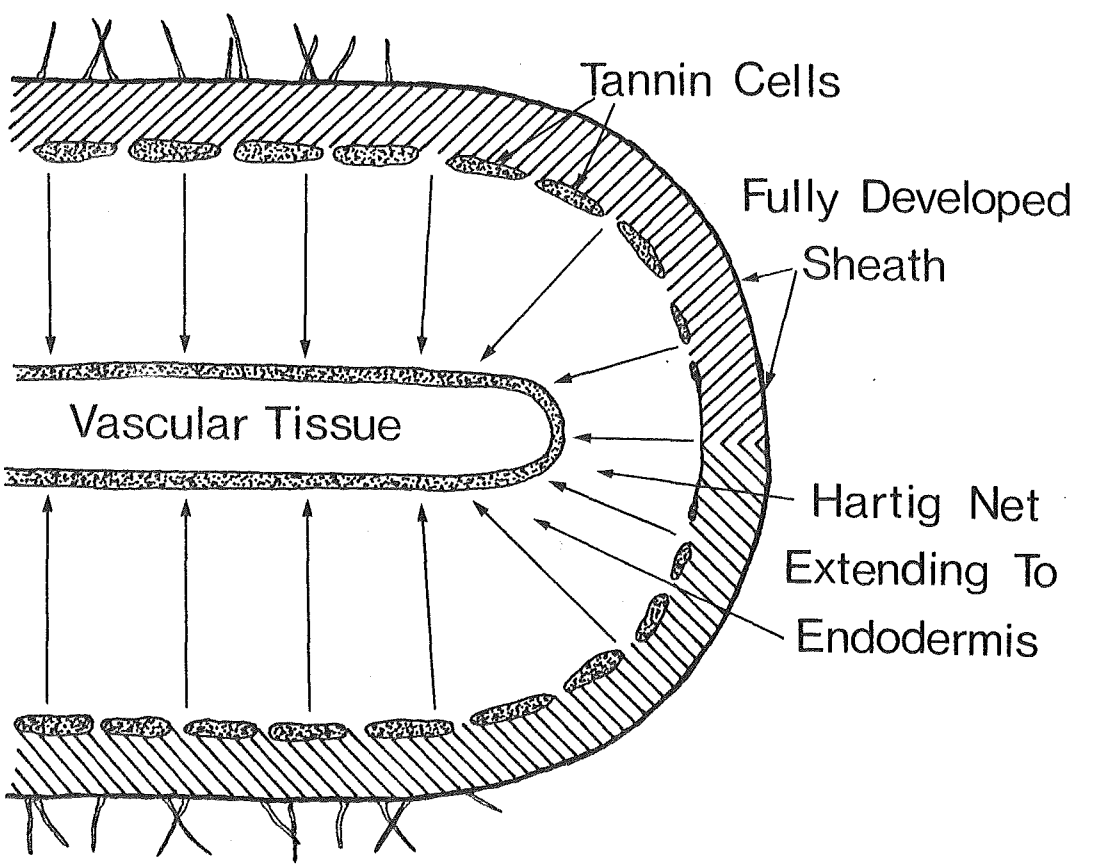


8-53

YOUNG, ELONGATING SHORT ROOT.



MATURE, NON-ELONGATING MYCORRHIZA.



As the mycorrhiza develops, deposition of insoluble polyphenols occurs, possibly as part of the host reaction to fungal invasion. The presence of 'vesicular bodies' in mycorrhizal roots may also be related to this reaction but the precise nature and function of these is unknown. It is unlikely that they are solely fungal in nature but if so they may well facilitate nutritonal exchanges between host and symbiont. They resemble in part the structures found in the ectendomycorrhizal type of association, although S. luteus has not been reported to form such mycorrhizas. They have been observed in mycorrhizal roots from seedlings raised in sterile soil inoculated with S. luteus basidiospores, thus eliminating the possibility of their being caused by other mycosymbionts of an ectendotrophic type. Their distribution is irregular in the outer layers of the cortical tissues and the percentage of cells containing them also varies considerably between short roots. If they were a type of storage body, a more regular occurrence of the 'vesicular bodies' would be expected. It is probable that they are of host origin, induced by the presence of the fungus in the root, although no particular cell type is affected. Structurally they are very similar to tyloses reported in the pathogenesis of resistant tobacco varieties by Verticillium dahliae (Mahanty, 1970) and in more recent S.E.M. work of this by the same author (unpublished). The 'vesicular bodies' may thus be a host reaction preventing penetration of its cells by the fungal hyphae.

CHAPTER IX

POLYPHOSPHATE STORAGE IN MYCORRHIZAL ROOTS

9.1 INTRODUCTION

Little is known of the fate of phosphorus once assimilated by mycorrhizal fungi. Much of the P appears to remain in the fungal sheath and less than ten percent can be detected in the host (Harley and McCready, 1952). Some plants are known to form insoluble phosphate salts (polyphosphates) in their roots and it is thought that this is a storage form of P (Jeffery, 1964; Nassery, 1968; Fernadez-Gomez *et al*. 1973). Recently polyphosphates have been identified in mycorrhizal roots of *Eucalyptus fastigata* by their metachromatic reaction with toluidine blue stain (Ashford *et al*. 1975; Ling-Lee *et al*. 1977). In eucalypt mycorrhizas, polyphosphate granules are located in the Hartig net and inner sheath hyphae. Ashford *et al*. (1975) consider that these granules are the primary storage form of the phosphorus taken up by the fungal symbiont and that in situations of phosphate deficiency, P from this source is remobilized and made available to the host via a small, soluble, orthophosphate pool in the fungal cytoplasm.

Phosphate availability, in soils such as those at Broken River, is of great importance to developing tree

seedlings. If polyphosphates are formed by S. luteus in its association with pines, adequate supplies of phosphorus should be available allowing for normal growth of the tree. Mycorrhizas of P. mugo/S. luteus were investigated to determine whether polyphosphates are formed in this association.

9.2 MATERIALS AND METHODS

Seedlings were raised as described in Section 8.2, in semi-aseptic culture (inoculated with basidiospores) and also in mycorrhizal duff infected soil. Mycorrhizal short roots were excised from the seedlings and kept in distilled water for the brief period before freeze microtome sectioning. Serial sections of many roots were made and subjected to the staining techniques given by Ashford et al. (1975). Toluidine blue was used at both pH 4.4 and 1.0, with or without an acid (0.1 N HCl) rinse. Sections were also stained with lead nitrate/ammonium sulphide and some were extracted with cold trichloroacetic acid (TCA) prior to staining.

9.3 RESULTS AND DISCUSSION

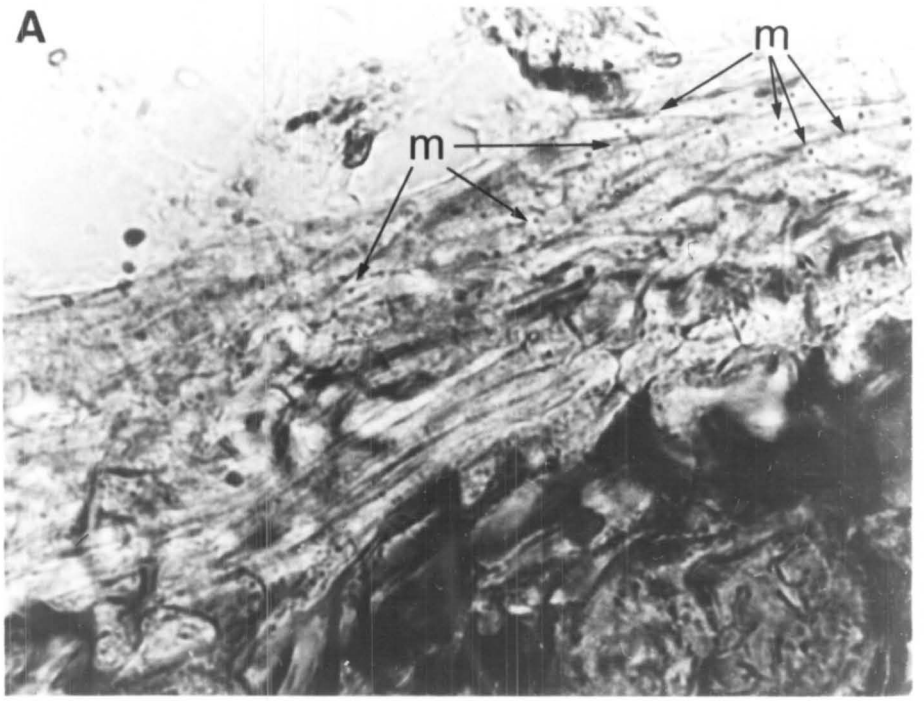
Metachromatic granules were detected in the mycorrhizal sheath and Hartig net of root sections stained with toluidine blue (pH 4.4, 1.0, or acid washed) and the lead reagent. Granules were present in both semi-aseptically cultured and naturally infected seedling roots (Fig. 9.1 a, b). They appeared to be more frequent in roots from sterilised soil,

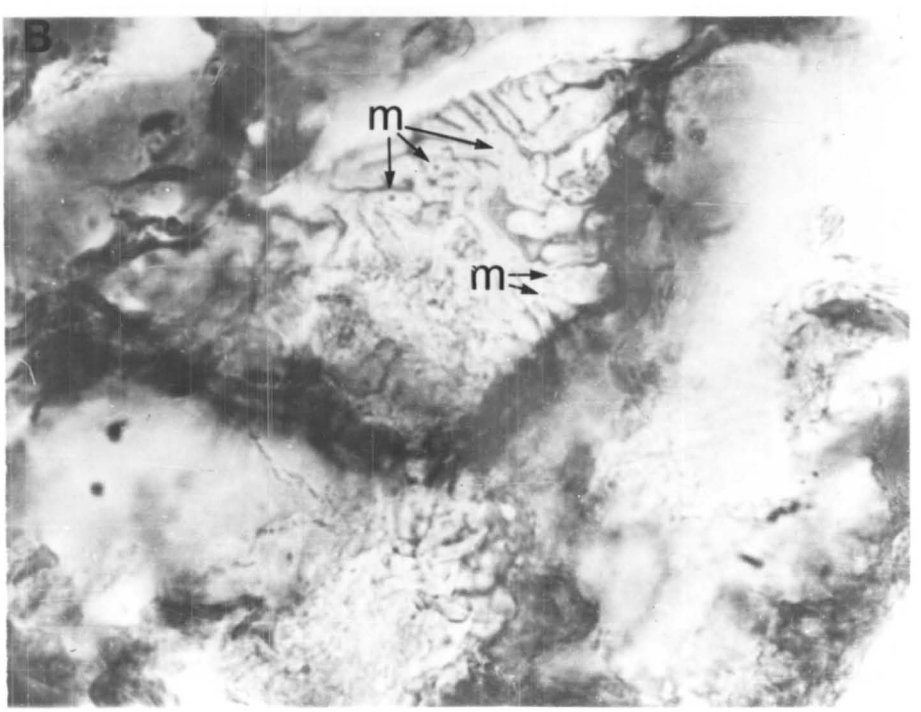
FIGURE 9.1 Polyphosphate presence in mycorrhizal roots of *P. mugo*.

A Metachromatic granules (M) visible in the sectioned sheath of roots from semi-aseptically cultured seedlings (inoculated with basidiospores). Acid toluidine blue stain. x800

B Metachromatic granules (M) present in Hartig net elements of seedling roots raised in naturally infected soil. Granules were infrequent in seedling roots raised in this way. x800

9-1 A





possibly caused by the release of additional phosphate into soil solution during the sterilisation process. TCA removed all trace of the granules, indicating that they contained polyphosphate rather than sulphated polysaccharides which also react with acid toluidine blue but are unaffected by TCA (Fig. 9.2). Granules were not found in control, unstained, mycorrhizal roots (Fig. 9.3).

The granules varied considerably in size and density, a feature also noted by Ashton et al. (1975), and thought to be correlated with the availability of phosphorus from the environmental. Ashton et al. also suggested that the granules are located in vacuoles but did not state whether these were of fungal or plant origin. This present study and results of Ling-Lee et al. (1977 plate 3; 11, 15) indicate that they are scattered in the fungal cytoplasm. The presence of polyphosphates would be an important factor in the survival of mycorrhizal trees in periods of phosphate deficiency. However, it is apparent that where soil levels of phosphate are low, as at Broken River, only limited amounts of P may be incorporated into this storage form. Most of the phosphate would be required by the tree for growth processes and thus is likely to be quickly converted via metabolism.

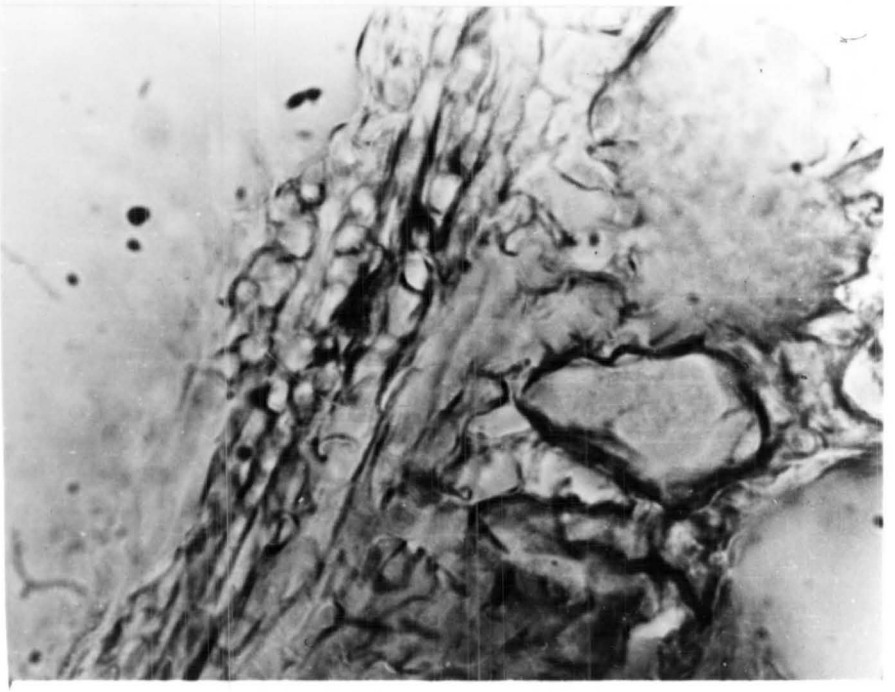
FIGURE 9.2 Root section stained with acid toluidine blue after extraction with trichloroacetic acid.

Polyphosphate granules, if present, are removed by this treatment but sulphated polysaccharides, which also stain with acid toluidine blue, remain. No granules are present in this micrograph confirming the presence of polyphosphate in the previous sections. x800

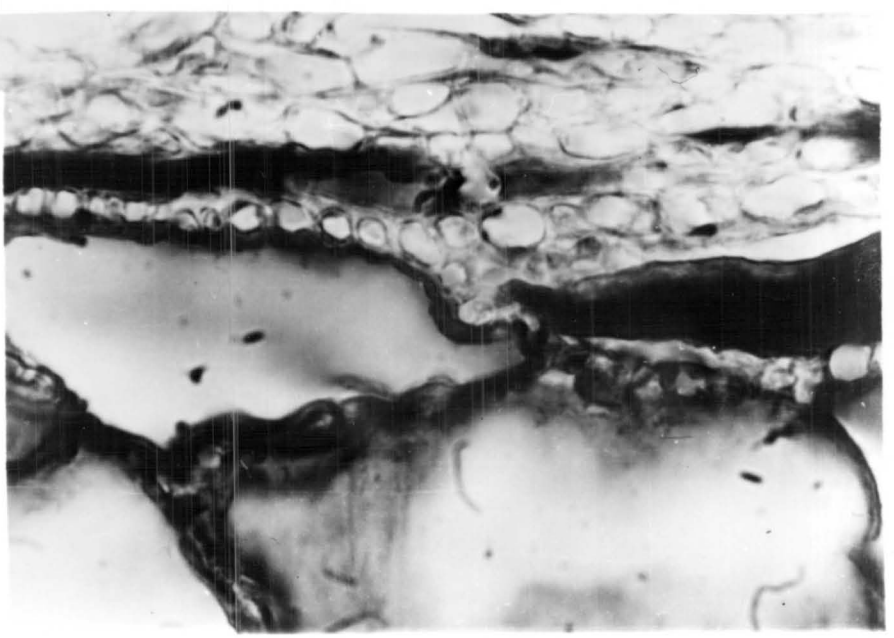
FIGURE 9.3 Control, unstained, mycorrhizal root.

No granules can be detected in the sheath hyphae. x800

9-2



9-3



CHAPTER X

GENERAL DISCUSSION AND CONCLUSIONS

Rapid tree establishment and conditions allowing maximal tree growth are required to ensure survival of revegetation species under harsh high altitude conditions. The seedling must, therefore, be able to commence growth early in the short growing season, it must have access to adequate levels of nutrients and it must have developed a stable root system before encountering frost heave and soil creep associated with winter conditions. Revegetation sites typically have poor, unstable soils often closely associated with the bedrock which, combined with the harsh climate, do not provide an environment conducive to tree growth. The association of seedling roots with a fungal symbiont (in the form of an ectomycorrhiza) provides access for the seedling to a supply of nutrients with the consequence that establishment and growth is more rapid. Of the several species of Suillus found in New Zealand, S. luteus seems to be best adapted to high altitude conditions. Pinus mugo is most suitable for revegetation work and the establishment of a mycorrhizal association between it and S. luteus was investigated to improve establishment and growth of the pine on revegetation sites.

S. luteus has the ability to grow well where levels

of phosphorus are low which is advantageous in the poor high country soils, as the P levels in these soils are limiting to tree growth unless mycorrhizal fungi are present. Phosphorus assimilated by the fungus can be stored in an insoluble form - polyphosphates - in the fungal sheath and Hartig net. The presence of these polyphosphates does, however, appear to be dependent on the availability of P in the soil. Where levels are low, metabolic conversion of phosphate into forms able to be assimilated by the tree is high and little is likely to be stored in polyphosphate form. Ammonium nitrogen sources were found to be good growth substrates for S. luteus and it is considered that, in the soil, the fungus may exist in close relationship with N-fixing bacteria, assimilating nitrogen from this source and thus aiding tree growth (Richards, 1973, 1974). S. luteus also responds positively to various organic acids and a wide range of mono- and dissacharides during growth on these substrates. Many of these are known constituents of plant root exudates (Theodorou and Bowen, 1973) and this may account for the wide host range of S. luteus (Trappe, 1962). Soil pH has a considerable effect on the establishment and type of mycorrhizal association and it was found that the optimum pH for growth of S. luteus is approximately 5.0. In litter layers under existing trees the pH was similar and the mycorrhizal mycelium was profuse, but in exposed soils the pH was more basic and no mycorrhizal mycelium was present.

To establish the association between P. mugo and S. luteus an effective source of mycorrhizal inoculum was necessary. Basidiospores were considered to be suitable as

an inoculum but many problems were found with their germination. In vitro, few spores germinated and it would seem that even under natural conditions few basidiospores are viable. Spores from different sporophores varied in their germination percentage and high spore densities also adversely affected spore germination. A number of substances were investigated for possible stimulatory effects on spore germination. Those stimulating germination were mycelial extracts (and the mycelium itself), a spore wash solution, urea and ammonium tartrate. Short wave ultraviolet light was also found to increase mycorrhiza formation from a spore inoculum. Nicotinic acid and inositol warranted further investigation in this respect. Both gave a high level of germination in comparison to that normally found with basidiospores and also stimulated mycorrhiza formation on seedlings from a spore inoculum.

The storage of spores from the time of fruiting body production until seed sowing, a period of six to seven months, poses further problems with maintenance of spore viability. Assessment of stored spores shows that viability decreases rapidly with long storage periods. Even after short storage periods it was found that the spores were difficult to germinate in vitro. A study of the ability of variously stored inocula to form mycorrhizas indicated that cool stored and frozen spores were best, followed by freeze dried spores and hymenial tissue. Similar results were shown when spores were stained with acridine orange and observed by fluorescent microscopy. When incorporated in a granulated seed, however, the freeze dried hymenial tissue (at a high rate of application)

gave good results. Although containing fewer and apparently less mature spores than the cool stored or freeze dried spore inocula, good infection of seedlings occurred with this inoculum. It also has advantages of ease of collection and preparation compared with pure spore inocula. When stained with acridine orange, the freeze dried hymenial inoculum showed greater numbers of live spores, which may account for its higher mycorrhiza production in the granulated seeds. Why more spores survive in this inoculum is unknown, but it may be related to spore retention on the sterigmata and the presence of spores at many different stages of maturity. Fully mature spores (released from the sporophore) show reduced viability after being freeze dried but survive well if stored under cool or frozen conditions.

Spore inocula gave best mycorrhizal formation when applied to seeds in a 'carrier' substrate such as sand and/or vermiculite, or soil, confirming the findings of Marx (1976). A definite lag period was consistently noted before mycorrhizas became apparent on seedlings, this increasing with low spore concentrations, extended storage times and low seed weights. The lag period must, therefore, depend on both spore germination and seedling development. Mycorrhizas are not commonly found until secondary and tertiary roots have become well developed and this may take a considerable period of time, particularly where the seedling is derived from a seed of low weight. Seedlings developing from heavy seeds (with larger quantities of endospermic food reserve) have clear advantages in establishment and growth, as well as

subsequent mycorrhizal development. Seed viability, like that of spores, was found to decrease with extended storage times, thus a fresh source of seeds and spores is required for inoculation purposes.

Once mycorrhizal infection is initiated, even if only on a small percentage of the short roots, further development and spread of the symbiont can take place and many more short roots will become mycorrhizal. Self sown seedlings growing at a distance from parent trees may survive several seasons (if not adversely affected by the harsh environment) before showing the growth increases signifying infection by a symbiont. Potential mycorrhizal short roots appear to be infected at an early stage in their development if the symbiont is present - possibly even before emergence from the mother root - but complete sheath development takes some time. The short root continues to elongate, during which time the Hartig net develops progressively between cortical cells, then stops and the sheath is completed. Tannin is also deposited in cells of the outer cortex at this time, possibly a reaction to fungal invasion. Also found in cells of the cortex are novel 'vesicular bodies'. These vary widely in abundance and form but appear to eventually coalesce and may cover the entire inner surface of the cell wall. They are closely associated with the Hartig net but not with any particular cell type, often resembling the structures observed in ectendomycorrhizal types of association. It is suggested that they may be of host origin, induced by the presence of the fungal symbiont and similar in nature to tyloses, structures found in

resistant plant species when attacked by pathogenic fungi. They may, therefore, protect the host cell against further invasion by the fungus.

Self sown seedlings may not become mycorrhizal for considerable periods of time thus it would appear that few other potential symbionts capable of forming mycorrhizas with pines are present in the high country soils. Competition between S. luteus and other symbionts would therefore be slight and dominance of the required association would be assured if an inoculum source is provided with the seed. Practical methods of achieving an inoculated seed were investigated and resulted in the development of a granulated seed. This forms a 'closed environment' in which both seed and spore germination can occur without hinderance. In the granulated seeds produced, slight reductions in seed germination occurred and they were not as effective at forming mycorrhizas as were spores applied in a 'carrier' but their effectiveness could be increased in several ways. Cool stored spores and freeze dried hymenial tissue could be used at greater concentrations to increase mycorrhiza formation. Chemical spore germination stimulants could be included in the seed coating and seeds may be selected for high viability and seed weight. The use of granulated seeds with a basidiospore type inoculum is a convenient way of providing the seedling with a selected fungal symbiont which not only colonizes the root and infects the plant rapidly but stimulates growth, thus satisfying the requirements of Bowen et al. (1971).

The addition of fertiliser with the inoculated seeds is not recommended as indicated by the detrimental effect it had on seed germination and mycorrhizal formation.
A similar effect is shown with the use of the fungicide thiram thus practical use of 'additives' such as these in seed coats is questionable.

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2 P. sylvestris + Rhizopogon rubescens.

3 ectendomycorrhizae of P. mugo + Boletus edulus and P. cembra + S. variegatus.

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APPENDIX 1 FUNGAL GROWTH MEDIA

All ingredient weights are per litre.

Medium

A M40 (Stevens, 1974)

Malt extract		5.0 g
Glucose		5.0 g
KH_2PO_4		0.5 g
$MgSO_4 \cdot 7H_2O$		0.5 g
NH_4Cl		0.5 g
Ferric citrate (1% soln)		0.5 ml
Distilled water		1000.0 ml
(Agar)		15.0 g
Supplements	Thiamine	50.0 µg
	Biotin	10.0 µg

B M40 A

As above but with ammonium tartrate substituted for NH_4Cl and Trace solution for Ferric citrate.

C PDA (Difco)

Potato infusion	200.0 g
Bacto-dextrose	20.0 g

D Trace solution

Ferric citrate	10.0 g
$ZnSO_4$	2.0 g
$CuSO_4 \cdot 5H_2O$	0.2 g
$MnSO_4 \cdot 4H_2O$	0.2 g
$Na_2MoO_4 \cdot 2H_2O$	0.2 g
Trace of H_2SO_4 to acidify	

E M.E. (Malt Extract)

Malt extract	5.0 g
Peptone	0.5 g
Glucose	2.0 g
Trace solution	0.5 ml
(Agar)	15.0 g
Thiamine	50.0 µg

F MP (Malt, Peptone)

Malt extract	2.0 g
Peptone	0.3 g
Trace solution	0.5 ml
(Agar)	15.0 g

G Glucose (from Santoro and Casida, 1959; ex Melin and Das, 1954).

Glucose	20.0 g
KH_2PO_4	1.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
Ammonium Tartrate	0.5 g
$ZnSO_4$ / Ferric citrate (1% soln)	see below
Thiamine	50.0 µg

Trace solution substituted for $ZnSO_4$/Ferric citrate (0.5 ml).

H Pine nutrient solution (Melin and Das, 1954)

KH_2PO_4	0.5 g
$CaCl_2$	0.05 g
NaCl	0.025 g
$MgSO_4 \cdot 7H_2O$	0.15 g
KNO_3	0.25 g
Trace solution	1.0 ml
Thiamine	50.0 µg

The following media (commercially obtained) were also used for growing S. luteus:

YM	yeast malt (Difco)
CM	corn meal (Difco)
*ME(B)	Bacto-Peptone (Difco)
*ME(C)	Casein hydrolysate (BDH)
*ME(P)	Proteose peptone (Oxoid)
*ME(S)	Soya peptone (Oxoid)

* Medium E(ME) with above ingredients substituted for peptone.

APPENDIX 2 *P. mugo* ROOT PREPARATION FOR SCANNING ELECTRON MICROSCOPY

Whole seedlings were carefully removed from trays or pots with the surrounding soil. The soil was then gently removed from around the roots without disturbing any obvious mycelium. The short roots were excised by scalpel and immediately placed in labelled specimen tubes containing the first fixative. If necessary, several washes in this were given to remove excess soil.

The preparation process was as follows:

FIXING

1 Fixation in 5% Glutaraldehyde in 0.1 M K-K 2 phosphate buffer (pH 7.0) overnight at 4°C.

2 Buffer rinses and washing - 24 hours.

3 Post-fixation in buffered 2% OSO_4 - overnight at 4°C.

4 Buffer rinse. Specimens cut with razor blade and some set aside as controls (non-hydrolysed).

HYDROLYSIS

1 2-3 minutes in 1% aqueous periodic acid.

2 Distilled H_2O rinses - 10 minutes.

3 Treat with 4% aqueous KOH, 30 minutes at 55°C. This was later modified to a 20 minute period due to the softness of the short root tissue.

4 5 minute treatment with 1% acetic acid.

5 Distilled H_2O rinses - 1 hour.

6 Buffered 2% OSO_4, overnight at 4°C.

7 Buffer rinses for both hydrolysed and control samples.

DEHYDRATION

Prepared roots were taken through a graded series to 100% ethanol, then to 100% amyl acetate in another graded series with a minimum time of 1 hour at each step.

NOTE: The specimens need not be infiltrated with amyl-acetate. Recent discussions have shown that this is not necessary for critical point drying and specimens can therefore be left in 100% ethanol.

PAPERS ACCEPTED FOR PUBLICATION DURING COMPLETION OF DOCTORATE DEGREE

1 B. J. WILLS and A. L. J. COLE (1977)

The use of mycorrhizal fungi for improving establishment and growth of *Pinus* species used for high altitude revegetation.

International Symposium on Microbial Ecology, Dunedin (N.Z.) August 1977.

2 B. J. WILLS and A. L. J. COLE (1978)

A scanning electron microscopy study of 'vesicular bodies' in mycorrhizal roots of *Pinus mugo* (Turra).

New Phytol. 80, 579-.

THE USE OF MYCORRHIZAL FUNGI FOR IMPROVING ESTABLISHMENT AND GROWTH OF PINUS SPECIES USED FOR HIGH-ALTITUDE REVEGETATION

Paper Presented at the International

Symposium on Microbial Ecology,

Dunedin, 1977.

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Botany Department, University of Canterbury.

INTRODUCTION:

Problems with the revegetation of high altitude terrain are posed by uncompromising climatic, edaphic and biotic conditions prevailing in these areas, particularly where reafforestation is proposed. Soil movement on slopes, frost heave, the short growing season and animal predation all result in high tree seedling mortality rates. For revegetation to be successful, early establishment and rapid growth of the seedling is essential. The development of a good mycorrhizal association is of great importance in this respect to fulfil these requirements.

This investigation is concerned with the problems of establishing effective mycorrhizal associations between *Pinus mugo* and *Suillus* (*Boletus*) *luteus* (L. ex Fries). Mountain pine is an exotic species found to grow successfully under N.Z. conditions up to 1600 m. It is resistant to wind and snow-pack damage, growing well on dry slopes. Its low spreading canopy makes it suitable for erosion control. *S. luteus* is believed to be the main mycorrhizal fungus associated with *Pinus* species at higher altitudes in N.Z. Because of its known association with *P. mugo* it was used in this study.

Pre-inoculation of seeds with the myco-symbiont would offer many advantages. The most effective association can be established between host and available symbionts, especially where the latter are absent from the soil. Viable ecto-mycorrhizas may thus be introduced to the revegetation sites with the seeds ensuring early establishment due to increased growth rates in the first season.

An effective mycorrhizal inoculum needs to be found and suitable methods of inoculation devised. Problems such as non-viability under field conditions, deterioration under extended storage times and insufficient supplies of the inoculum source must, however, be overcome.

Previous work (Moser, 11, 12, 13; Gobl, 3) has shown pure cultured mycelial inocula to be successful. If growing conditions are stable or can be controlled, as in the nursery or growth room, this method is effective (Hacskaylo, 4; Bryan and Zak, 1; Trappe, 20; Lamb and Richards, 5; Shoulders, 15; Riffle, 14). Success of such inoculations under severe conditions has not been evaluated, but deterioration of inoculum in the field and the vast quantities needed limit its use.

Little work has been done to develop a basidiospore inoculum and information is lacking on basidiospore germination and viability. Spore germination levels are characteristically low with many of the mycorrhizal basidiomycetes. Levels of spores must often exceed 10^6 or 10^7 per seed before mycorrhizal associations are established (Marx and Ross, 10; Theodorou, 18; Theodorou and Bowen, 19; Marx, 9). Where spores are stored or freeze dried, these levels must be increased 10 or 100 fold to be effective.

MATERIALS AND METHODS:

Seeds of P. mugo were supplied by the N.Z. Forest and Range Experimental Station, Rangiora, from collection sites at Broken River (Canterbury). They were surface sterilized in 30% Hydrogen

Peroxide for 1 hr and washed in sterile distilled water.

S. luteus sporophores are produced from February until April. Collections were made throughout this season and spores were dropped on to large glass sheets by placing the inverted pileus on these overnight. The spores (representing a mixed sample of 20-30 sporophores) where then scraped into Bijou bottles and stored at 4° C or freeze dried.

Soil was obtained from Broken River and sterilised for 4 hrs at 85° C. It is a High Country Yellow Brown Earth (pH 6.0), with high total Phosphorous and high Phosphorous retention.

RESULTS AND DISCUSSION:

Many attempts have been made to induce germination of various basidiospores in vitro. Losel (7, 8) obtained germination of Agaricus bisporus spores by stimulation with living mycelium and also by isovaleric acid. Fries (2) mentioned difficulties in obtaining germination with several Boletus species but using an activator organism, such as Rhodotorula, the spores germinated on a malt extract medium. On a medium (M40) modified from Stevens (17), S. luteus spores have germinated in this laboratory when incubated at 25° C - initial germination of a few spores tends to overcome the dormancy in adjacent spores. The period of incubation varied from 2-4 weeks and only occurs when fresh spores, dropped from sporophore tissue on to the agar surface, are used. Spores stored for any length of time have not been germinated successfully. Average germination percentage figures in vitro are as low as 0.1% and the yeast activator had no effect. Stack et al. (16) could not germinate spores of Laccaria laccata

in vitro but obtained a 46% inoculation level of Douglas fir seedlings in pot trials. Lavallee and Lortie (6) achieved a 30% germination level for spores of *Pholiota aurivella* after 5 days on malt-extract agar, and the spores maintained their viability for 6 months.

Several possible germination stimulants were tested by adding sterile solutions to the modified M40 agar. Of those tried, citric acid, $CaHPO_4$ and Malt Extract had some effect. Inositol and Nicotinic Acid stimulated germination sufficiently to warrant further investigation (Table 1) but stored spores have not germinated with these yet. During these tests it was noted that a definite spore concentration effect was evident. In areas of spore drops from hymenial tissue where spore counts exceeded 1000 per sq. mm, no spores germinated. Germination was most prevalent at concentrations of 500-600 per sq. mm. Marx (9) has postulated a water soluble germination inhibitor with P. *tinctorius* and a similar effect is possibly being observed here.

Although difficulties are encountered with *in vitro* studies, pot trials have shown that the spores are still viable. Viability is difficult to assess. Stack et al (16), using acridine orange and fluorescent microscopy, determined that 25% of L. *laccata* spores were still viable and could produce mycorrhizas on Douglas fir seedlings. Spores of S. *luteus* were mixed with a known volume (50 cc) of the carriers (sand, vermiculite, soil and distilled water) which were then added to the top of soil filled pots (10 cm of diameter). Seedlings grown in these were assessed for mycorrhizal short root development at monthly intervals and show

in Graphs I and II. 0.1 g and 0.01 g spore levels correspond to 3.6×10^8 and 3.6×10^7 (± 9%) total numbers of spores respectively. It can be seen that a definite lag period occurs before the mycorrhizal association is formed, this is more pronounced at the 0.01 g spore level. This may be because of the time taken for production of lateral roots, on which the mycorrhizal short roots are borne. Seedlings take 2-3 weeks to emerge and another 3-5 weeks before the secondary laterals become well established. Additional delays may be caused by slow spore germination and subsequent contact between root and mycelium. The initial mycorrhizal infection is restricted to a zone 2-3 cm in radius around the primary root and about 1 cm in depth below the root collar. At both spore concentration levels, the sand and vermiculite carriers provide a better inoculum source, soil being less effective. The inhibitory effect of a spore suspension in distilled water is evident. With the onset of fungal penetration to form the Hartig Net, the tips of short roots swell and become translucent in appearance. During this period the dry weight of the mycorrhizal seedling shoot increases steadily as compared to the controls. Control root systems are, however, more extensive than the mycorrhizal ones. This might be expected if the fungus was largely supplementing the absorbtive role of the plant root system.

The ability of the spore inoculum to form mycorrhiza's was studied at different spore concentrations. A dilution series

of spores in distilled water was made and used to inoculate seeds. After 3 months the seedlings were assessed for mycorrhizal associations (Table 2). This shows that numbers of mycorrhizal short roots increase steadily up to the maximum level (10^8 spores) used. Some seeds were coated with spores in the same trial but these gave poor results. This may be due to hypocotyl extension and subsequent raising of the testa out of the soil. Many spores will be taken with it and the few remaining may be insufficient to allow mycorrhizal development.

SUMMARY:

The use of basidiospores of Suillus luteus to provide an effective mycorrhizal inoculum source for application to Pinus mugo seeds has been investigated. In vitro germination of basidiospores was very low but additions of inositol and nicotinic acid stimulated germination. Mycorrhizal development in seedlings was enhanced when sand and vermiculite were used as basidiospore carriers. A concentration of 10^8 spores in the carrier medium was most effective in mycorrhizal short root formation.

TABLE I EFFECT OF CHEMICAL STIMULANTS ON BASIDIOSPORE GERMINATION

Treatment	% Germination ≥ 0.1%	Treatment	% Germination <0.1%
Nicotinic Acid	0.96	NAA	0.06
Inositol	0.85	$CaHPO_4$	0.06
Citric Acid	0.19	Adenine HCl	0.03
Malt Extract	0.16	Fumaric Acid	0.03
Control	0.00	EDTA	0.02
		Thiamine	0.02
		Pimelic Acid	0.02

TABLE II EFFECT OF FRESH SPORE CONCENTRATION ON MYCORRHIZA FORMATION

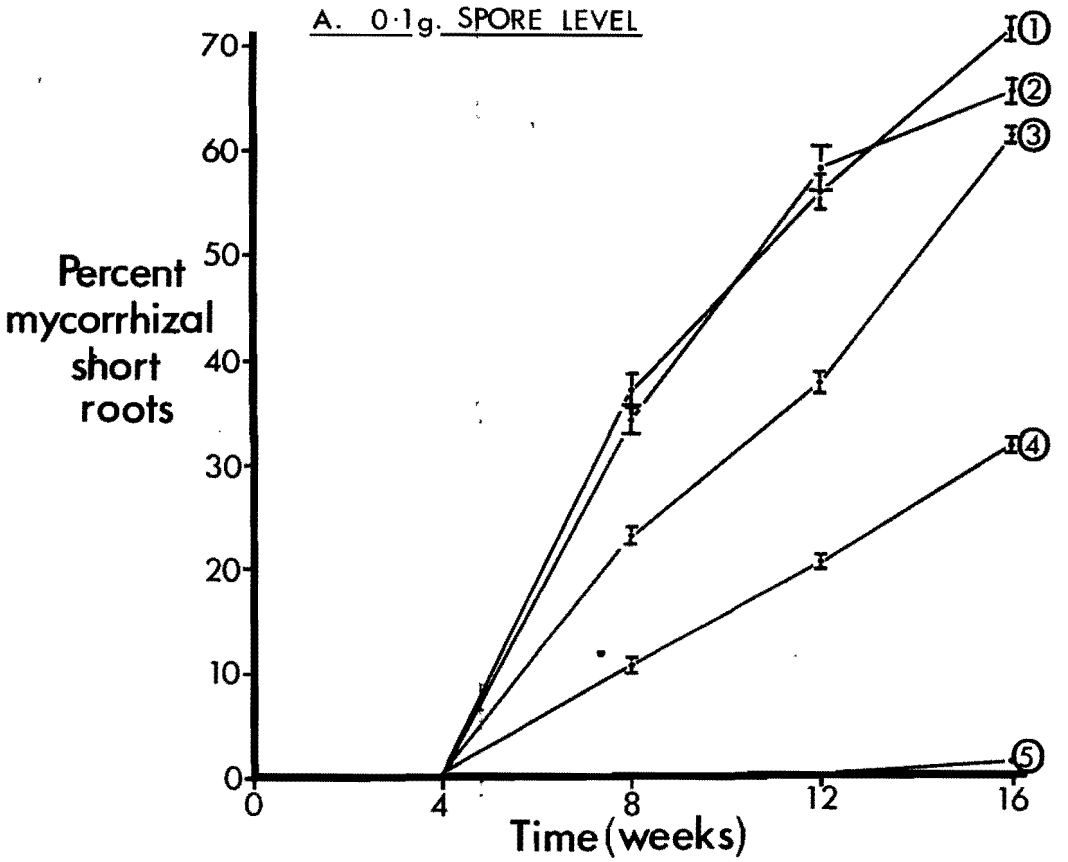
Spore Concentration/Seed			10^4	10^5	10^6	10^7	10^8
Percent Mycorrhizal Short Roots per Plant	Spore Aliquot	A	0	7.8	21.3	31.7	46.0
		B	0	47	47	80	87
	Slurry Coated Seeds	A	2.4				
		B	33				
	Control		0				

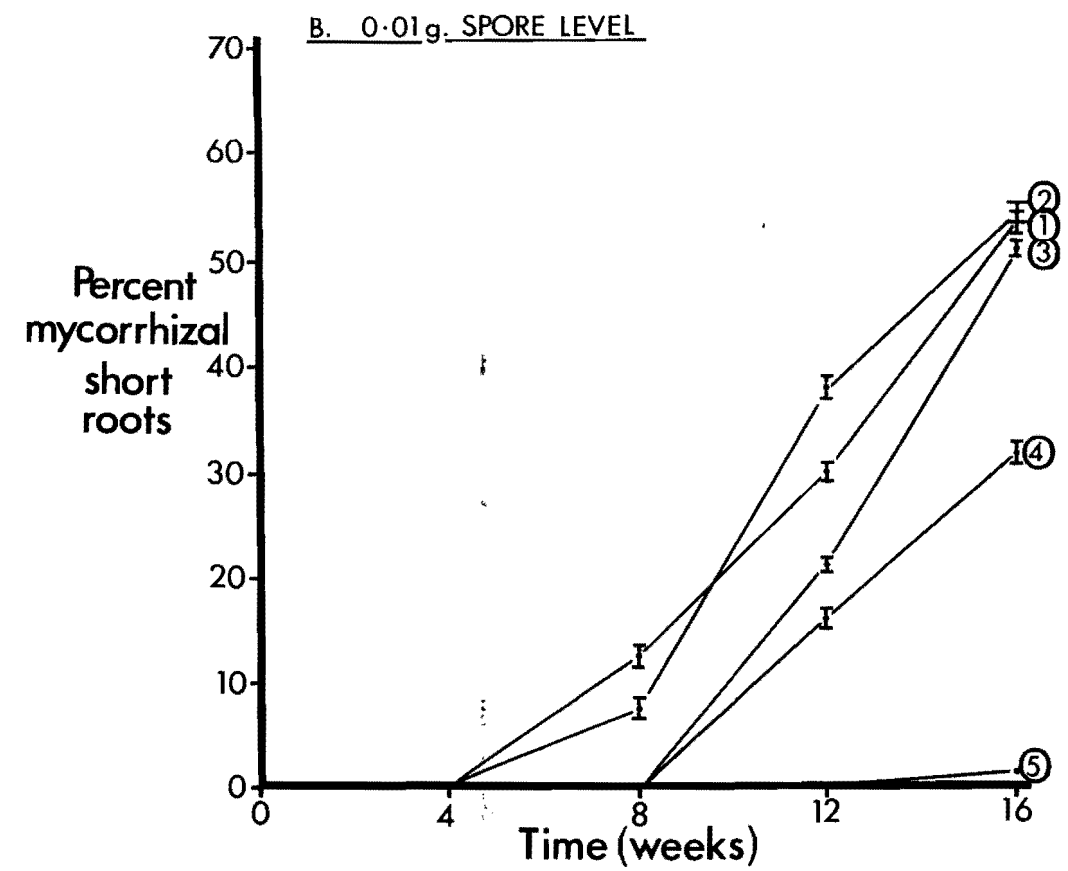
A % mycorrhizas on infected seedlings.

B % of assessed seedlings showing mycorrhizal infection.

GRAPH I, II

Effect of spore 'carriers' on mycorrhiza formation.





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A SCANNING ELECTRON MICROSCOPY STUDY OF 'VESICULAR BODIES' IN MYCORRHIZAL ROOTS OF *PINUS MUGO* (TURRA)

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SUMMARY

The formation of vesicular bodies in mycorrhizal short roots of *Pinus mugo* has been investigated using the scanning electron microscope. They appear to bear a relationship to the Hartig net but their exact nature is as yet unexplained.

INTRODUCTION

Conifer mycorrhizal root systems have been studied by numerous workers, including a detailed light microscopic study of *Pinus* spp. mycorrhizae by Hatch and Doak (1933). Investigations on the diversity of mycorrhizal form in pines with different mycosymbionts were made by Laiho (1965), Mikola (1965) and Wilcox (1968). Root colonization was shown to vary from the ecto-mycorrhizal association to ectendo- and pseudo-mycorrhizal forms. Transmission electron microscope (T.E.M.) studies have revealed the close association between host and mycosymbiont in the balanced mycorrhizal situation (Hofsten, 1969; Strullu, 1976). Apart from the recent studies of yellow poplar endomycorrhizas by Kinden and Brown (1975a, 1975b, 1976), few comprehensive investigations of mycorrhizas using the scanning electron microscope (S.E.M.) have been made.

An S.E.M. study of mycorrhizal colonization of Mountain Pine (*Pinus mugo*) by the bolete *Suillus luteus* (L. ex Fries) has shown the presence of 'vesicular' bodies in roots of colonized seedings. In this paper an investigation of the siting and nature of these novel bodies is described.

MATERIALS AND METHODS

Soil was obtained from a forested site 1100 m above sea level in the Craigieburn Range, North Canterbury, New Zealand, in which the main symbiont of mountain pine at high altitudes, *Suillus luteus*, was present. Seedlings of *Pinus mugo* were grown in this soil. Soil was also sterilized by heating to 180°C for 4 h and in this both control seedlings and seedlings inoculated with *Suillus luteus* basidiospores were grown. Roots from 6 to 24-month-old seedlings showing a well-developed mycorrhizal sheath and dichotomous root branching were excised and prepared for S.E.M. by the method of Kinden and Brown (1975). Control roots from sterile soil and non-colonized roots from untreated soil were prepared in an identical manner. After critical point drying specimens were mounted on aluminium S.E.M. stubs using double-sided adhesive tape, coated with gold in an Edwards 306 vacuum coating machine and examined with a Cambridge Stereoscan 600 S.E.M. operating at 15 or 25 Kv.

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RESULTS AND DISCUSSION

In non-mycorrhizal roots the characteristic root cap protecting the meristematic region is clearly observed as shown in Plate 1, No. 1 but in mycorrhizal short roots the root cap is absent and the root tip has a rounded appearance (Plate 1, No. 2). An extensive sheath of fungal hyphae is visible covering the root surface and elements of the Hartig net are seen between cells of the cortical tissue. At the extreme root tip only a narrow region of outer cortex is invaded by sheath hyphae; however, at 0.25-0.5 mm from the tip of the root, hyphal elements can be seen to penetrate to the endodermis. The vascular tissues have been partially removed and granules (10 μm diameter) are evident. Sections stained with Lugol's or Gram's Iodine indicate that these are amyloplasts in cortical cells adjacent to the vascular tissue. At greater magnification cells of the outer cortical layer of short root tips are seen to contain 'vesicular bodies' which are much smaller than the amyloplasts (Plate 2, No. 3). They do not give positive reactions in staining tests for polysaccharides.

The prevalence of these 'vesicular bodies' in short roots is extremely varied. Some roots have few cells containing the inclusions but in others they are prolific as shown in Plate 2, No. 4. A large variation in density of the bodies between adjacent cells of the same root is often observed (Plate 2, No. 4). Two types of vesicle can be distinguished. The predominant type shown in Plate 3, No. 5, have a convoluted appearance in contrast to the less frequently found smooth type (Plate 3, No. 6). The convoluted vesicles range in size from 2 μm to 6 μm in diameter. Fewer of these large vesicles are found per cell. With increasing size there appears to be a change in shape from nearly spherical to a more irregular outline. They may occasionally cover the entire inner surface of the cell wall. It appears that the large vesicles may be formed by the coalescing of two or more smaller ones as seems to be indicated in Plate 3, No. 5 and Plate 5, No. 9 (*). The surface features of the vesicles closely resemble the warted appearance of the fungal hyphae (Plate 2, No. 4). The smooth vesicles are of a similar size range but are predominantly of 2-3 μm diameter. They also appear to coalesce but have not been observed as the large irregular forms that are found with the convoluted type. Neither type of vesicle is present in control roots.

The close spatial relationship of both smooth and convoluted vesicles to the Hartig net is evident in these micrographs. They appear to be either attached to the cell wall or are projections from Hartig net hyphae passing through the cell wall. The vesicles can be dislodged, as shown in Plate 4, No. 7, thus they may be a form of adcrusting deposit similar to that reported by Zee (1975) in wheat caryopsis. In some cells they are sited on a membranous-like structure in close apposition to the cell wall and appear to be closely associated with intracellular tannin deposits shown in pine root cells by Hofsten (1969) and in T.E.M. studies in this laboratory (unpublished work). Plate 4, No. 8 reveals differences between adjacent cells, one containing small (3-4 μm diameter) convoluted vesicles and the other large irregular convoluted vesicles. Again their close relationship to the Hartig net elements is clearly visible and the vacuolate nature of the vesicles evident in Plate 5, No. 9 would indicate their structure to be more consistent with that of a modified fungal hypha than a host storage body. A similar feature can be seen in Plate 5, No. 10 in a cell containing irregular convoluted vesicles. A portion of one vesicle's wall has been removed (arrow) and there appears to be continuity with the fungal hyphal network.

The precise nature and function of these vesicles is unknown. If they are fungal in nature they may well facilitate nutritional exchanges between host and symbiont. They resemble in part the structures found in the ect-endomycorrhizal type of association although *Suillus*

luteus has not been reported to form such mycorrhizas. They have been observed in mycorrhizal roots from seedlings raised in sterile soil inoculated with S. *luteus* basidiospores, thus eliminating the possibility of their being caused by other mycosymbionts of an ect-endotrophic type. If host in origin they may be induced by the presence of the fungus in the root although they are not restricted to any particular cell type. Their distribution is irregular in the outer layers of the cortical tissues and the percentage of cells containing them also varies considerably between short roots. If they were storage bodies, a more regular occurrence of the vesicular bodies would be expected.

They may be characteristic of *Pinus mugo* when colonized by this particular symbiont. Further studies are in progress to ascertain this using different pine species and fungal symbionts.

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EXPLANATION OF PLATES

Key to Symbols: R = root cap; M = meristematic region; S = sheath (fungal); H = Hartig net; V = 'Vesicular body'; C = cortex; F = fungal hypha; G = granule; * = coalescing vesciles.

PLATE 1

No. 1. Section through non-mycorrhizal root-tip showing characteristic root cap protecting the meristematic region. ×160.

No. 2. Section through mycorrhizal root-tip showing the fungal sheath, Hartig net and large granules (10 μm diameter) adjacent to vascular tissue. Hatched line indicates approximate penetration of hyphae into cortex. ×160.

PLATE 2

No. 3. Cells in mycorrhizal root-tip with 'vesicular bodies' present in the outer cortex. ×707.

No. 4. Cortical cells showing prolific 'vesicular bodies' and variations in density and size of these between adjacent cells (a and c). The bodies have a similar appearance to the sheath hyphae (b). 1 and 3 × 682.5; 2 × 658.

PLATE 3

No. 5. Convoluted vesicles showing their close proximity to the Hartig net. Coalescing vesicles are evident. ×3360.

No. 6. Smooth vesicular bodies. ×3570.

PLATE 4

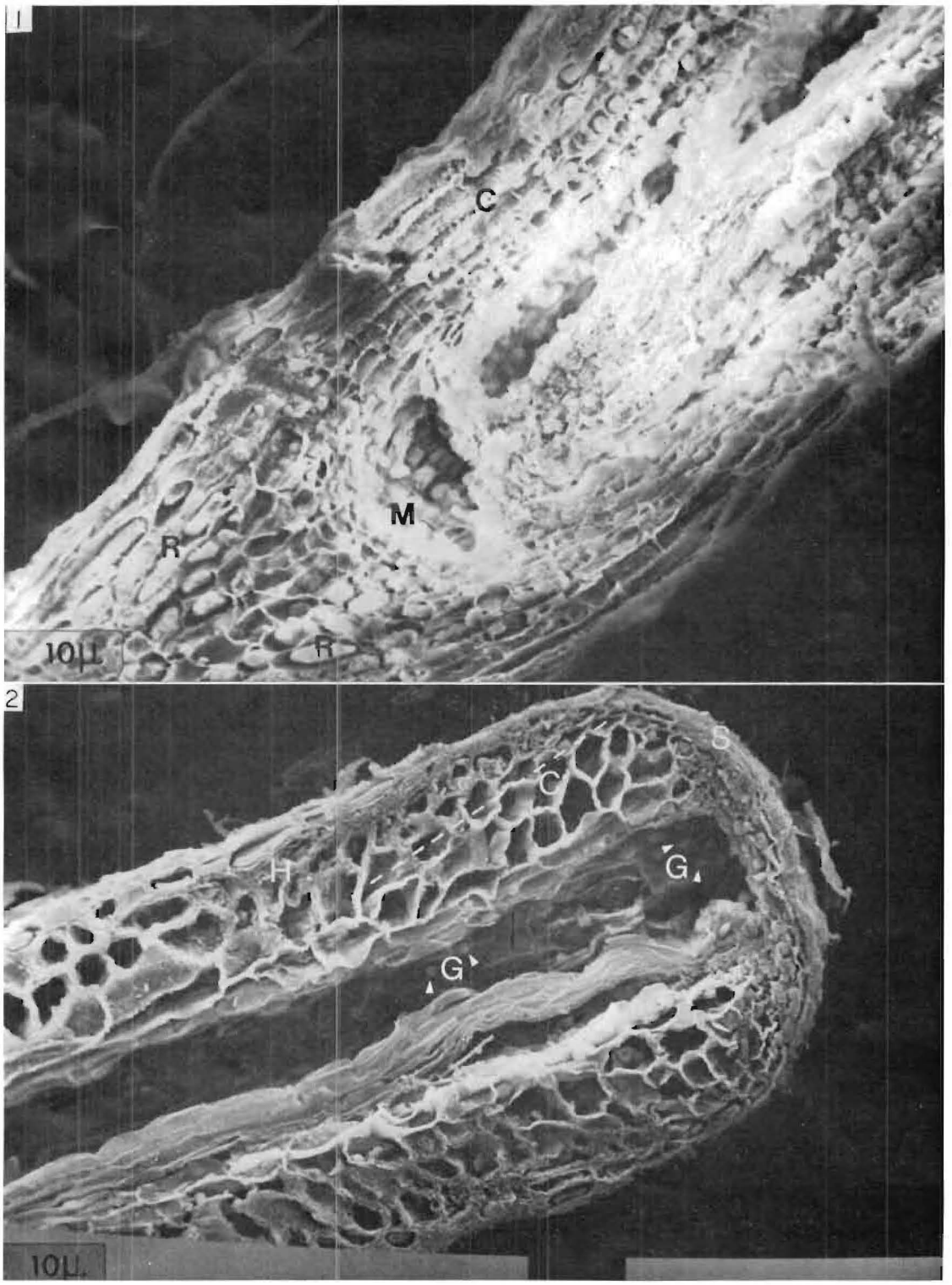
No. 7. Cell containing convoluted vesicles, some having been dislodged from the inner cell wall surface (arrows). ×2855.

No. 8. Large and small convoluted vesicles found in adjacent cells, with the Hartig net between. ×3225.

PLATE 5

No. 9. The vacuolate nature of the vesicle in the lower portion of the micrograph is evident. Coalescing of vesicles can also be seen. ×3225.

No. 10. Section of a cell containing irregular convoluted vesicles adjacent to the Hartig net. Part of the wall of one vesicle has been removed (arrows) and continuity with the fungal hyphal network is apparent. ×3225.



B. J. WILLS and A. L. J. COLE—*VESICULARBODIES IN PINE MYCORRHIZAS*

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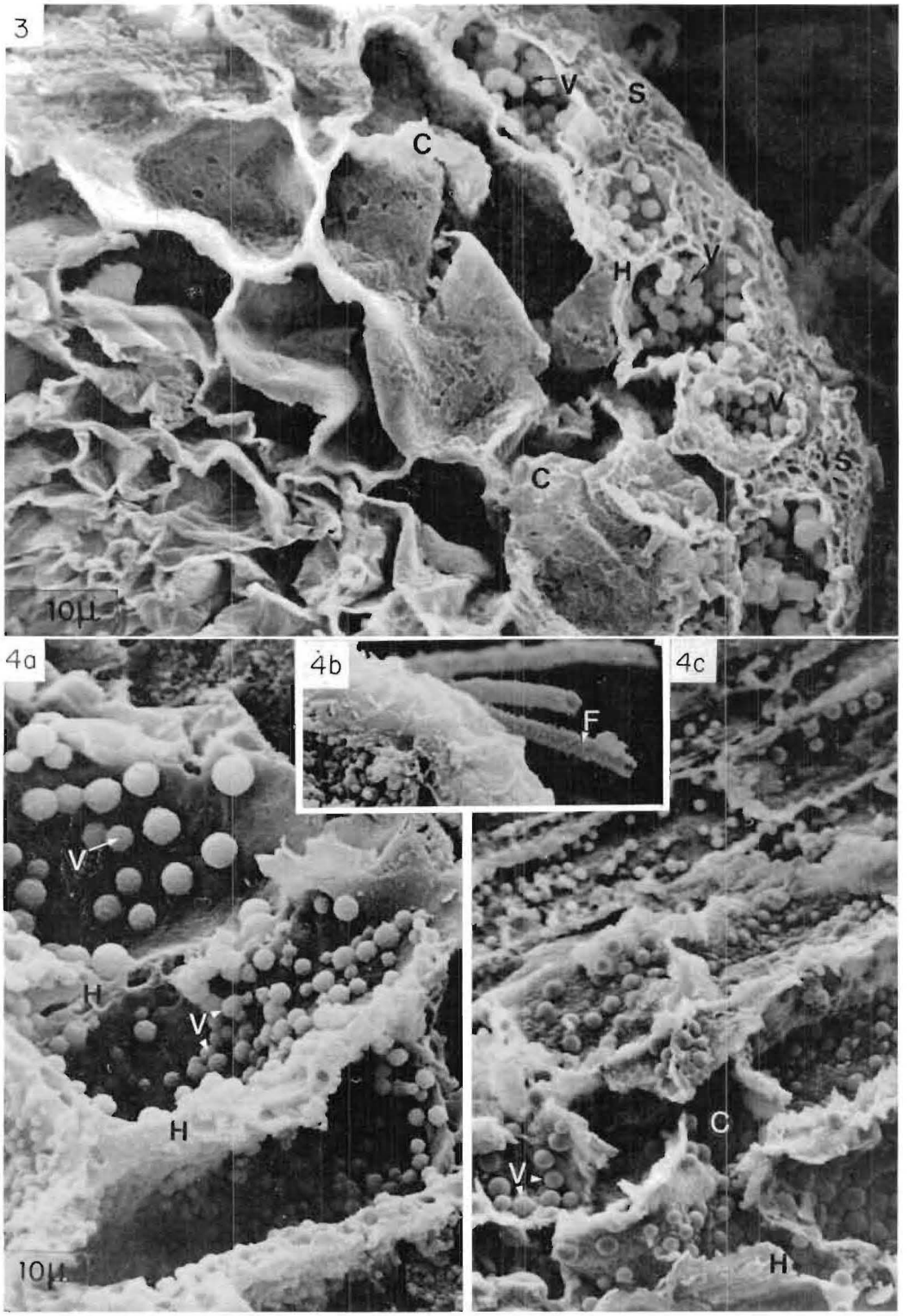

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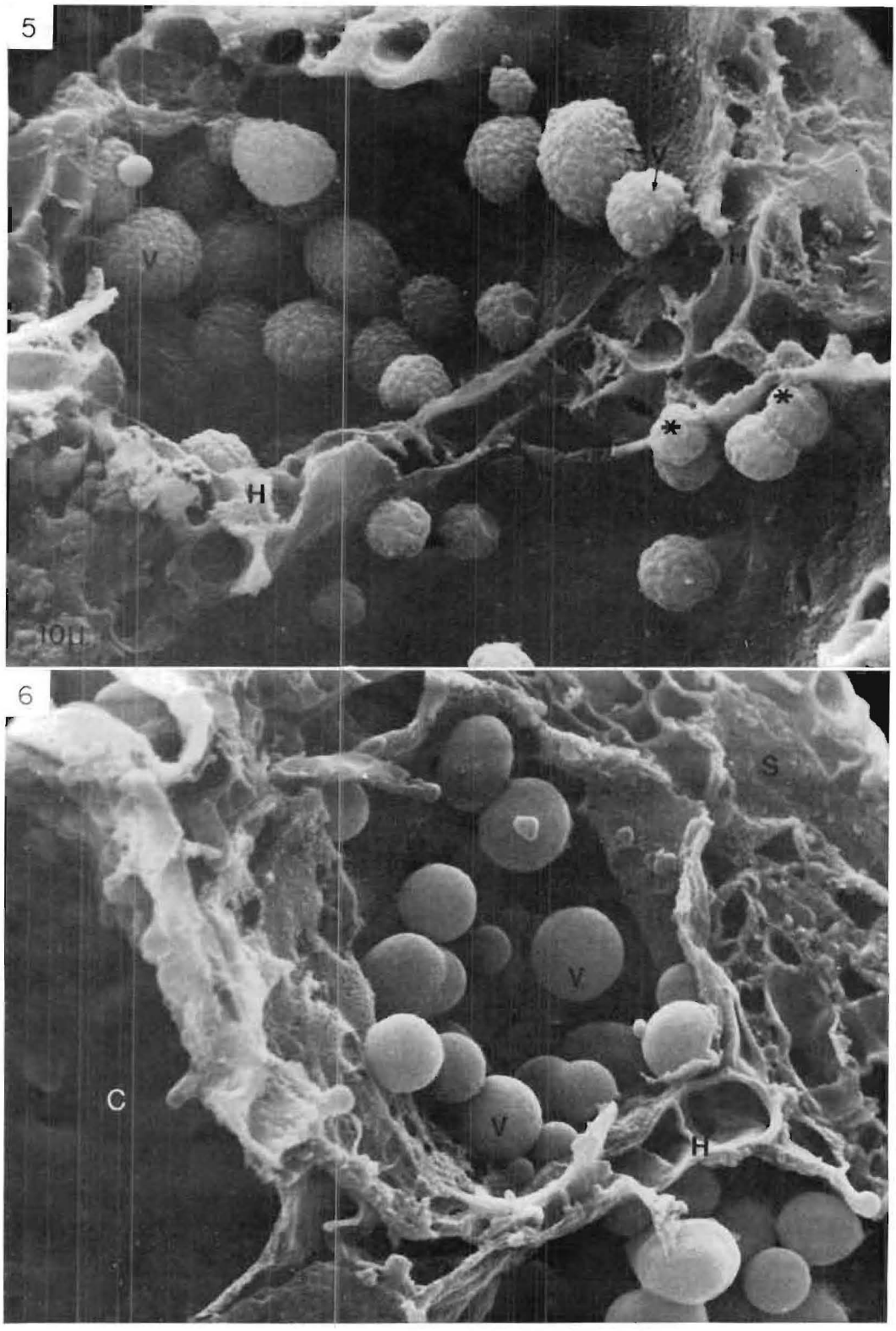

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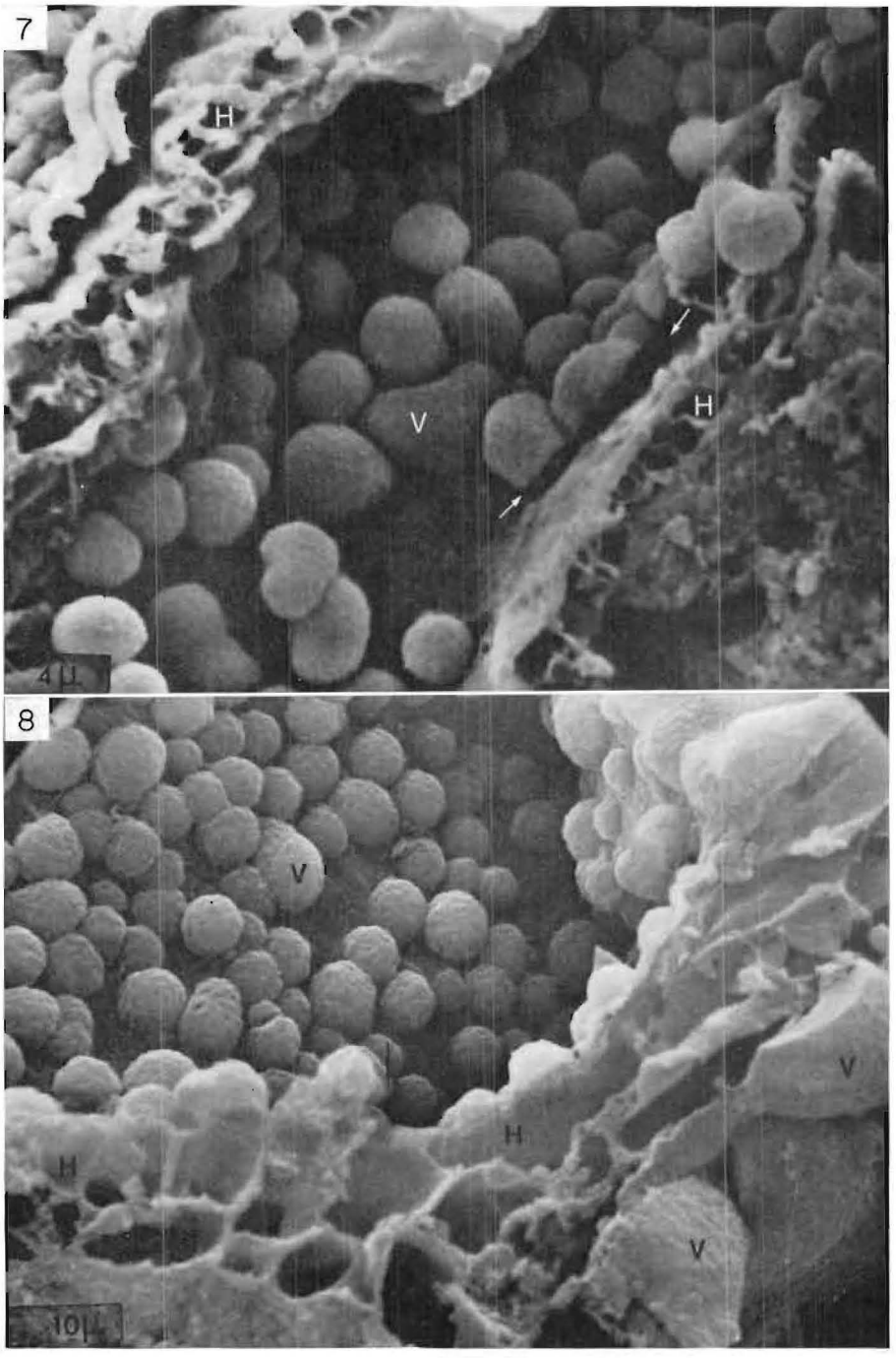
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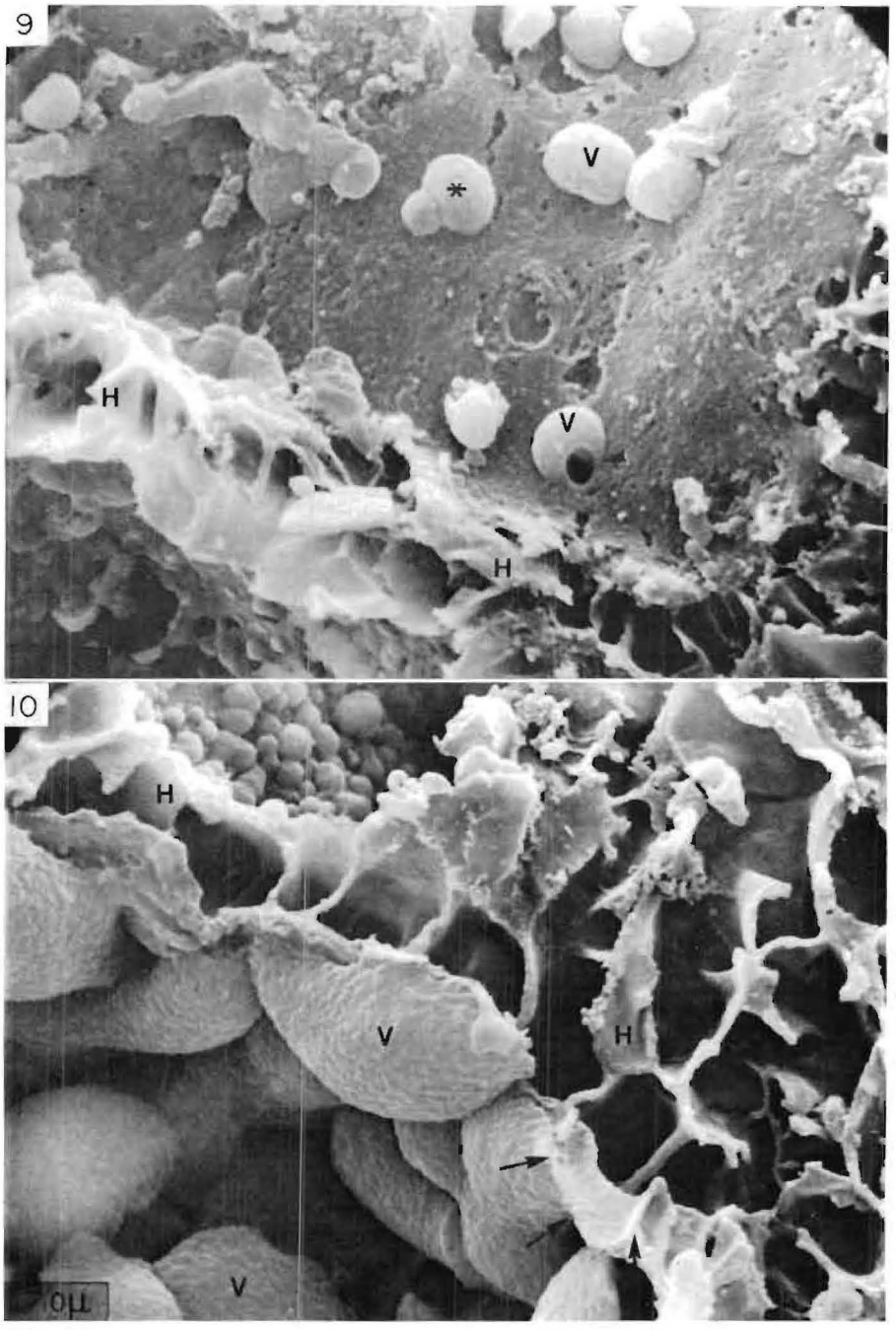

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